Hydrogen Sulfide (H2S) and the Cardiovascular System

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Abstract

Hydrogen sulfide (H_2S) has relatively recently been added to a list of endogenously produced gaseous signalling molecules. Our understanding of the science of H_2S has advanced rapidly in recent years as exemplified by the fact that, within a mere 10 years, a range of H_2S releasing drugs have already been synthesisied and some indeed are entering clinical trials. However, the precise biological roles of endogenous H_2S are not fully understood. In this respect, slow releasing H_2S donors, such as GYY4137, have played a part in elucidating the complex roles of this gas in the body and are also beginning to show promise as possible therapeutics in inflammation - an area in which the function of H_2S remains ambiguous.

This thesis attempts to provide some additional clarity to the biological significance of endogenous H_2S. The first part of this work examines the release of endothelial cell derived H_2S *in vitro* and the consequences of deleting the genetic encoding of nitric oxide synthase on tissue H_2S biosynthesis in mice. As part of this study, I show that the methods currently utilised to measure H_2S are insufficiently sensitive/reliable to convincingly demonstrate the release of H_2S from cells *in vitro*. In addition, data reported herein has demonstrated that knocking out endothelial cell nitric oxide synthase (eNOS) results in an increase in tissue H_2S synthesising activity, which is hypothesised to be a compensatory mechanism as a result of the loss of NOS.

The second part of this thesis examines the role of H_2S in inflammation and provides further evidence for its anti-inflammatory activity both *in vitro* and *in vivo*. In addition, this thesis has shown the 'added benefit' of slow-releasing H_2S donors (c.f. conventional sulfide salt based donors) in that the H_2S released from slow-releasing

donors is progressive and does not instantaneously expose cells to potentially cytotoxic amounts of H_2S. In response to the need for additional slow releasing H_2S donors, attempts were made to examine the H_2S releasing ability and antioxidant capacity of a library of additional compounds. As a result of this work, a novel compound, ZJ802 was shown to exhibit more potent antioxidant ability than the current commercially available H_2S donors. Furthermore, ZJ802 was shown to exhibit anti-inflammatory activity *in vivo*.

Overall, the roles of H_2S in physiology are not yet clear. There is scope for improvement in the current methods available to detect H_2S. Thus, the necessity for pharmacological tools, such as slow releasing H_2S donors and selective inhibitors of enzymes involved in H_2S synthesis, cannot be over-emphasised. Whilst the possible use of H_2S donors in the clinic has been raised there is still a need for more detailed preclinical, pharmacokinetic and long term drug safety and toxicological studies.

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Publications

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List of Figures

List of Tables

Contents

List of Abbreviations

AOAA	Aminooxyacetic acid
ATP	Adenosine-5'-triphosphate
BAEC	Bovine aortic endothelial cells
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Ca^{2+}/CaM	Calcium-calmodulin
CBS	Cystathionine beta-synthase
CSE	Cystathionine gamma-lyase
CO	Carbon monoxide
COX	Cyclooxygenase
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid)
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FCS	Fetal calf serum
GIT	Gastrointestinal tract
GYY4137	Morpholin-4-ium-4-methoxyphenyl(morpholino) phosphinodithioate

h	Hour
H_2O	Water
H_2S	Hydrogen sulfide
Hb	Haemoglobin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO	Haem oxygenase
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
IκB-α	Inhibitory protein κB-α
IL-1β	Interleukin-1β
ICAM-1	Intracellular adhesion molecule-1
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
i.pl	Intraplantar
ISE	Ion selective electrode
i.v.	Intravenous
KO	Knock-out
L-NAME	NG-nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
M199	Medium-199
MCh	Methacholine
Min	Minutes
MPO	Myeloperoxidase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

mV	Millivolts
Na_2S	Sodium sulfide
NaHS	Sodium hydrogen sulfide
NF-κB	Nuclear factor-κB
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
P5'P	Pyridoxal-5'-phosphate
PAG	DL-propargylglycine
p.o.	Per os (oral administration)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
SAM	S-adesosylmethionine
SAH	S-adenosylhomocysteine
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SNAP	*S*-nitroso-*N*-Acetyl-D,L-Penicillamine
SNP	Sodium nitroprusside
TCA	Trichloroacetic acid
Temp	Temperature
TFP	Trifluoperizine
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumour necrosis factor-α

VCAM-1	Vascular cell adhesion molecule-1
v/v	Volume to volume
WT	Wild type
w/v	Weight to volume

1. Introduction

1.1. Chemistry of hydrogen sulfide (H_2S)

Hydrogen sulfide (H_2S) is a colourless and flammable gas with a characteristic smell of rotten eggs. In solution H_2S dissociates into the hydrosulfide anion (HS^-) and the sulfide dianion (S^{2-}):

$$H_2S \rightleftharpoons HS^- + H^+ \rightleftharpoons S^{2-} + 2H^+$$

The majority of methods used to measure H_2S, detect molecular H_2S, HS^- and S^{2-} (Olson 2009). Therefore, in this report, the definition of "H_2S" will include the sum of all free sulfide species. The proportion of H_2S that dissociates into HS^- or S^{2-} depends on the pH of the solution. An acidic solution will shift the equilibrium to the left and an alkali solution will shift the equilibrium to the right. Intrinsically H_2S is a weak acid and has an acid dissociation constant (pKa) of 6.76 at 37^oC (Dombkowski, et al. 2004). In physiological solutions, such as plasma or extracellular fluid, approximately 80% of the dissolved H_2S spontaneously hydrolyses into HS^- and the other 20% remains as molecular H_2S (Dombkowski, et al. 2004). The cell cytoplasm is slightly more acidic, with a pH range between 7.1 – 7.4 (Bright, et al. 1987). Therefore within the cell, the ratio of H_2S:HS^- is calculated to be approximately 1:2 (Olson 2009).

HS^- is capable of conversion to S^{2-} (pKa_2 11.96) but only 2.8 x 10^{-3} % of H_2S is present in this form at pH 7.4 (Ondrias, et al. 2008) therefore making it virtually non-existent in biological tissues (Olson 2009). A larger concentration of S^{2-} may be found in the small intestinal mucosa, where alkali bicarbonate (HCO_3^-) is secreted to neutralise stomach acid from pH 1.1 – 1.7 (Lui, et al. 1986, Dressman, et al. 1990) to an intestinal

luminal pH of 5.9 - 7.6 (Bown, et al. 1974, Evans, et al. 1988). However, even then, a pH change from 6 to 8 would only increase S^{2-} by a 100 fold (Ondrias, et al. 2008).

1.2. Toxicity of H_2S

H_2S is found naturally from volcanic emissions, hot springs and from the bacterial catabolism of organic matter (Li, et al. 2009a). Micromolar levels of H_2S are found in the soil of marshes as a result of anaerobic metabolic activities of bacteria that use sulfur as electron acceptors (Bouillaud and Blachier 2010). H_2S is also synthesised during industrial processes such as: the manufacture of pulp in paper mills (Jaakkola, et al. 1990); from tanneries during the processing of leather (Boshoff, et al. 2004); during coal liquefaction (Parka, et al. 1998); sewage treatment (Costigan 2003); and oil refineries (Kemper 1966). H_2S has also been described as a toxin or poison due to its association with a number of occupational deaths (Baldelli, et al. 1993).

In the UK, the current safe occupational exposure for H_2S is 5 ppm (8 h time weighted average) and 10 ppm (15 min, short term exposure limit) (Costigan 2003). The threshold for the perception of the H_2S odour is approximately 0.02-0.13 parts per million (ppm) (Costigan 2003). An exposure of H_2S >100 ppm can lead to the impairment of smell, and is a key factor leading to the over exposure and inhalation of this gas (Dorman, et al. 2002, Costigan 2003). Exposure to different concentrations of H_2S can lead to a number of symptoms: H_2S >50 ppm can lead to eye and airway irritation; H_2S 250-500 ppm can lead to pulmonary oedema and headaches; H_2S 500-1000 ppm results in nausea and ataxia; H_2S >1000 ppm will lead to inhibition of the

central respiratory drive and death (Beauchamp, et al. 1984, Oesterhelweg and Puschel 2008).

Over exposure of this gas is particularly prominent in the oil and gas industry where H_2S is a large component of natural gas (Baldelli, et al. 1993). Molecular H_2S is lipid soluble and is capable of crossing biological membranes (Mathai, et al. 2009). Once H_2S crosses the membrane barrier and dissociates into HS^- and H^+, HS^- is capable of inducing multiple effects in the cell.

1.2.1. H_2S and cytochrome c oxidase inhibition

Mitochondria produce chemical energy in the form of adenosine-5'-triphosphate (ATP) necessary for normal cellular respiration. To produce ATP, mitochondria must maintain a membrane potential driven by protons (H^+). This H^+ membrane potential is maintained by the electron transport chain in the mitochondria. The generation of an electrochemical H^+ gradient in the mitochondrial membrane drives the phosphorylation of adenosine diphosphate (ADP) into ATP (Bouillaud and Blachier 2010). The primary mechanism by which H_2S induces its toxic effects is thought to be through direct inhibition of cytochrome *c* oxidase (complex IV), the ultimate enzyme in the electron transport chain of mitochondria (Beauchamp, et al. 1984). Cytochrome *c* oxidase is composed of two haem groups (*a* and a_3) and two copper centers (Cu_A and Cu_B) (Pietri, et al. 2010). Haem a_3 and Cu_B make up the catalytic centre of cytochrome *c* oxidase, and H_2S has been demonstrated to modify these two catalytic subunits, leading to the (reversible) inhibition of cytochrome *c* oxidase (Pietri, et al. 2010). Moreover, exposing rats to a sublethal concentrations of

H_2S (50-400 ppm) for 4 h can lead to a significant reduction in the activity of lung cytochrome *c* oxidase (Khan, et al. 1990).

In addition, H_2S has been demonstrated to induce apoptosis in a number of cell types: H_2S (200 μM) human aortic smooth muscle cells (Yang, et al. 2004), H_2S (100 μM) INS-1E insulin-secreting β-cells (Yang, et al. 2007), H_2S donor sodium hydrogen sulfide (NaHS, 10 μM) pancreatic acinar cells (Cao, et al. 2006), H_2S donor sodium sulfide (Na_2S, 500 μM) periodontal cells (Zhang, et al. 2009), and NaHS (250 μM) lymphocytes (Mirandola, et al. 2007). Several mechanism for H_2S-induced apoptosis have been proposed including: activation of p38 MAPK (mitogen activated protein kinase) (Yang, et al. 2007), activation of ERK1/2 (extracellular signal-regulated kinase 1/2) (Yang, et al. 2004), activation of caspase-3, -8, or -9 (Yang, et al. 2004, Cao, et al. 2006, Li, et al. 2009c), the induction of Bax expression (Cao, et al. 2006), inhibition of Bcl-2 expression (Li, et al. 2009c), and endoplasmic reticulum stress (Yang, et al. 2007).

Remarkably, H_2S is also capable of inhibiting apoptosis: NaHS (1 mM) HCT116 colon cancer cells (Rose, et al. 2005), NaHS (100 μM) pancreatic β-cells with (Taniguchi, et al. 2010), NaHS (1 – 300 μM) SHSY5Y neuroblastoma cell line (Hu, et al. 2009), H_2S inhalation (80 ppm) retinal cells (Biermann, et al. 2011), NaHS (100 μM) MC3T3-E1 osteoblastic cells (Xu, et al. 2011), NaHS (1 – 100 μM) cardiomyocytes (Hu, et al. 2008b), Na_2S (1 mg/kg) hepatocytes (Jha, et al. 2008) and NaHS (1.83 mM) neutrophils (Rinaldi, et al. 2006). Suggested protective anti-apoptotic mechanisms include: preservation of mitochondrial function (Hu, et al. 2009), inhibition of p38 MAPK (Hu, et al. 2009, Sivarajah, et al. 2009, Xu, et al. 2011), inhibition of JNK (c-jun N-terminal kinase) (Hu, et al. 2009, Biermann, et al. 2011, Xu, et al. 2011), activation of ERK1/2 (Hu, et al. 2008b, Biermann, et al. 2011), inhibition of ERK1/2 (Xu, et al. 2011),

activation of PI3K/Akt (phosphatidylinositol 3-kinase/protein kinase B) (Hu, et al. 2008b), K_{ATP} channel activation (Hu, et al. 2008b, Sivarajah, et al. 2009), attenuation of caspase-3, -9 activity (Sivarajah, et al. 2009, Biermann, et al. 2011), increasing HSP-90 (heat shock protein-90) expression (Jha, et al. 2008, Biermann, et al. 2011), and increasing Bcl-2 expression (Jha, et al. 2008).

Indeed the reason for the disparity between H_2S induced apoptosis through the ERK1/2 pathway is unclear. However this is not a H_2S specific phenomenon. ERK1/2 activation has been demonstrated to have anti-apoptotic effects in response to TNF-α (Tran, et al. 2001), hypoxia (Buckley, et al. 1999), sodium nitroprusside (Kim, et al. 2002), radiation (Park, et al. 1999) and other types of stimuli (Lu and Xu 2006). In contrast, ERK1/2 activation has also been demonstrated to promote apoptosis in response to a number of stimuli including ultra violet and ionising irradiation (Tang, et al. 2002), glutamate (Stanciu, et al. 2000), TNF-α (Cheng, et al. 2008), sodium nitroprusside (Gomez-Sarosi, et al. 2009) and others (Cagnol and Chambard 2009). The reason behind the difference may be dependent on the length of time of its activation (that is, transient or prolonged activation), cell type, the stimuli and/or cell culture conditions. Indeed, the mechanism by which ERK1/2 activation promotes or inhibits apoptosis requires additional clarity.

Although H_2S is thought to exert its toxic effects through inhibiting cytochrome *c* oxidase, interestingly, this is also thought to be the mechanism by which H_2S induces suspended animation in mice (Blackstone, et al. 2005). Mice exposed to H_2S have a reduction in core temperature and metabolic rate compared to control (Blackstone, et al. 2005, Volpato, et al. 2008). Moreover, mice pre-conditioned with H_2S (150 ppm, 20 min) are capable of surviving for several hours in lethal hypoxia (5% v/v O_2) compared

to control mice which die within 20 min (Blackstone and Roth 2007). However, the relevance of these finding to larger mammalian species is uncertain, as most are unable to dramatically drop their core body temperature within a short period of time. In anesthetised, mechanically ventilated piglets (Li, et al. 2008a) and sedated spontaneously breathing sheep (Derwall, et al. 2011), inhalation of H_2S (20 – 80 ppm) did not reduce total carbon dioxide production or increase oxygen consumption (endpoint markers for aerobic respiration), suggesting H_2S does not alter metabolism in larger animals.

Interestingly, low micromolar (<2 μM) concentrations of H_2S are capable of energising the mitochondria by donating electrons to the respiratory chain (Goubern, et al. 2007). This is thought to occur during the oxidation and detoxification of sulfide to sufate (see later) (Hildebrandt and Grieshaber 2008).

1.3. Endogenous detoxification mechanisms for H_2S

Although H_2S can inhibit mitochondrial activity, mitochondria are sites where the detoxification and removal of H_2S takes place. In the body, the process of H_2S oxidation and detoxification occurs predominantly in the liver and colon, and subsequently excreted in the urine (Levitt, et al. 1999). The primary route by which H_2S is removed in cells is through oxidation to sulfate (SO_4^{2-}) or thiosulfate ($S_2O_3^{2-}$) (Hildebrandt and Grieshaber 2008). In mitochondria, sulfide (S_2^-) is oxidised to elemental sulfur (S^o) via sulfide quinine oxidoreductase (SQR) a mitochondrial membrane flavoprotein, resulting in the formation of SQR-persulfide (SQR-SSH) (Hildebrandt and Grieshaber 2008). During this process, electrons released from this process are fed into

ubiquinone pool further driving the generation of ATP from oxidative phosphorylation (Hildebrandt and Grieshaber 2008). Persufide groups (e.g. SQR-SSH or reduced glutathione (GSSH)) are further oxidised to sulfite (SO_3^{2-}) by sulfur dioxygenase present in the mitochondrial matrix (Hildebrandt and Grieshaber 2008, Kabil and Banerjee 2010). Sulfite is then directly oxidised to sulfate by sulfite oxidase and excreted in the urine (Kabil and Banerjee 2010). Alternatively, sulfite is converted to thiosulfate by sulfur transferase, and excreted or further converted to sulfate by thiosulfate reductase (Hildebrandt and Grieshaber 2008).

A secondary route for H_2S detoxification is though cytosolic methylation by thio-methyltransferase to form methanethiol (CH_3SH) and dimethylsulfide (CH_3SCH_3) (Beauchamp, et al. 1984). This reaction occurs prominently in the liver and the intestinal mucosa (Beauchamp, et al. 1984).

Other miscellaneous ways of removing free H_2S is through binding to metals or reacting with free radicals. H_2S is capable of reacting with a number of metals to form metal sulfides. It is therefore likely H_2S may also react with a number proteins that contain metal complexes, for example metalloproteins, such as haem and cytochrome *c* oxidase (Peterson, et al. 1995). Oyxgen (O_2) can be metabolised to give rise to a number of chemically reactive compounds including both free radicals and non-radical forms (Halliwell 1987), collectively known as reactive oxygen species (ROS). H_2S is capable of reacting with a number of ROS such as peroxynitrite ($ONOO^-$) (Whiteman, et al. 2004), nitric oxide ($^{\bullet}NO$) (Whiteman, et al. 2006), hydrogen peroxide (H_2O_2) (Laggner, et al. 2007), and hypochlorous acid (HOCl) (Whiteman, et al. 2005, Laggner, et al. 2007). However, the rate constants for these reactions are currently unknown. It has also been suggested that activated neutrophils are capable of metabolising H_2S to

sulfite as a result of ROS formation from activated NADPH oxidase (NOX) (Mitsuhashi, et al. 2005).

1.4. Existence of endogenous H_2S

Interestingly, despite the toxicological profile of H_2S, this gas is endogenously synthesised and utilised by a number of prokaryotic and eukaryotic cells. In the deep sea where there is limited oxygen and sunlight is absent, bacteria oxidise sulfur compounds to obtain energy (Bouillaud and Blachier 2010).

In the human large intestine, millimolar concentrations of H_2S (1 – 2.4 mM) have been reported, thought to be the result of the breakdown of sulfurous compounds in the diet by gut flora (Macfarlane, et al. 1992). Sulfur is also a vital component of human dietary intake. Dietary intake of sulfur-containing amino acids is essential for the normal function of a number of proteins. L-cysteine, L-methionine, and L-taurine are sulfur-containing amino acids (Brosnan and Brosnan 2006). However, only L-cysteine and L-methionine are incorporated into proteins (Brosnan and Brosnan 2006).

Taurine is the most abundant free amino acid and acts as an antioxidant through a mechanism that is neither through scavenging free radicals or modulating antioxidant defense systems (Jong, et al. 2011). Interestingly, depleting taurine in neonatal cardiomyocytes results in impared electron transport and increased superoxide production by mitochondira, hypothesisied to be as a result of impairing the synthesis of mitochondrial proteins essential in assembling respiratory chain complexes (Jong, et al. 2011).

L-methionine is a hydrophobic essential amino acid and is the initiating amino-acid in protein synthesis for most eukaryotic proteins (Brosnan and Brosnan 2006). L-methionine can also be irreversibly converted to L-cysteine via the transulfuration pathway (Brosnan and Brosnan 2006). L-cysteine is essential in maintaining tertiary structure of proteins through forming disulfide bonds between adjacent cysteine residues (Brosnan and Brosnan 2006). Apart from protein structure and function, L-cysteine is also important for the generation of endogenous H_2S and glutathione (GSH) (Williamson, et al. 1982, Griffith 1999).

Research on cysteine desulfuration and the formation of H_2S in the liver and kidney have been ongoing for several decades (Stipanuk and Beck 1982). Warenycia and colleagues (1989) (Warenycia, et al. 1989) were the first to discover that H_2S was produced endogenously in rat brain with levels of 1.57 ng/mg brain tissue. A decade later it was demonstrated that endogenous H_2S production is not restricted to the central nervous system but also occurs in the cardiovascular system (Hosoki, et al. 1997). A few years later, others confirmed their observations, that H_2S is produced endogenously within blood vessels, and research on vascular H_2S was commenced in earnest (Zhao, et al. 2001).

Since 2001 appreciable micromolar concentrations of H_2S were described in the plasma and in a number of other tissues. Reported plasma levels range from 2 µM (Olson 2009) to 280 µM (Han, et al. 2006), with most studies reporting a plasma concentration ~30-45 µM H_2S (Zhao, et al. 2001, Zhong, et al. 2003, Yang, et al. 2008). However, more recently, the development of a new polarographic sensor, that measures H_2S gas in real time (without the need for harsh chemical modification of H_2S) suggests that this gas is undetectable in plasma (that is, below the detection limit

of 100 nM H_2S) (Whitfield, et al. 2008). This finding has recently questioned whether H_2S exists as a free circulating gasotransmitter. Despite these concerns, H_2S is currently thought to take part in a number of signalling processes necessary for normal physiological function (see later) (Yang, et al. 2008).

Thus the biological role of H_2S bears similarity to the history of $^{\bullet}NO$ and carbon monoxide (CO), which were initially thought of solely as environmental pollutants and toxins and later discovered to be synthesised by mammalian cells and elicit important biological effects. Together, H_2S, $^{\bullet}NO$ and CO belong to a family of gaseous endogenous molecules known as gasotransmitters (Wang 2002a). $^{\bullet}NO$ was the first of the gasotransmitters to be recognised subsequently followed by CO and then H_2S (Wang 2002a). Gasotransmitters are signaling molecules, like neurotransmitters and other mediators in the body, but have gaseous properties (Wang 2002a). All gasotransmitters are small-molecule, cell-permeable gases which are produced endogenously and are able to exert functional effects at physiologically relevant concentrations (Wang 2002a) and are thought to be synthesised at the sight of action (Oess, et al. 2006).

1.5. Endogenous production of H_2S

The endogenous production of H_2S occurs enzymatically in the cytosol of cells primarily by two pyridoxal-5'-phosphate (P5'P, an active form of vitamin B_6) dependent enzymes known as cystathionine β-synthase (CBS, Enzyme Commission [EC] number 4.2.1.22) and cystathionine γ-lyase (CSE, also known as γ-cystathionase, EC 4.4.1.1).

CBS is found on chromosome 21 (21q22.3) in humans (Gene ID: 875), chromosome 17 in mice (Gene ID: 12411) and chromosome 20 (20p12) in rats (Gene ID: 24250). CBS is a homotetrameric enzyme with at least 5 mRNA isoforms, of which isoform 1 and 2 are most abundant (Bao, et al. 1998, Kraus, et al. 1998). Each subunit has a molecular mass of 63 kDa and contains prosthetic groups for P5'P, S-adesosylmethionine (SAM) and haem (Kery, et al. 1994, Kraus, et al. 1998). An estimated four P5'P cofactors bind to each subunit, and removal of half results in loss of CBS activity (>94%) (Taoka, et al. 1999). The allosteric activator SAM binds to the C-terminal regulatory domain of each CBS subunit (Kery, et al. 1998), and increases its activity by 3 fold (Finkelstein, et al. 1975). The activity of CBS is also redox active, as oxidation of haem from Fe(II) to Fe(III) results in a 2-fold increase in activity (Taoka, et al. 1998).

CSE, like CBS, is a homotetrameric enzyme with a P5'P binding site on each subunit (Clausen, et al. 1998, Messerschmidt, et al. 2003, Sun, et al. 2009). There are approximately 31% (126 amino acids) conserved sequences between CSE and CBS, with a total homology of 36% including acceptable amino acid replacements (Belfaiza, et al. 1986). In humans, CSE is located on chromosome 1 (1p31.1) (Gene ID: 1491), in mouse this enzyme is located on chromosome 3 (Gene ID: 107869) and in rat on chromosome 2 (2q45) (Gene ID: 24962). This enzyme has a subunit molecular mass of 43.6 kDa (Ishii, et al. 2004). At least two isoforms of CSE mRNA have been detected, but it is likely these are splice variants rather than products of different genes (Levonen, et al. 2000).

CBS is widely thought to be the primary source of H_2S in the brain, whereas CSE was thought to be the primary source of H_2S in the peripheral tissues. This conclusion

has arisen from publications demonstrating relatively low levels of CSE and high levels of CBS in brain tissue (Abe and Kimura 1996, Ishii, et al. 2004). Moreover, CSE was predominantly found in the liver, kidney, gastrointestinal tract (GIT), smooth muscle cells and the vascular endothelium (Zhao, et al. 2001, Ishii, et al. 2004, Yang, et al. 2008) and as such, CSE is thought to be the predominant enzyme in the cardiovascular system. As such, many publications have focused on CBS in the central nervous system, whereas work within the cardiovascular field has predominatly focused on the activity and expression of CSE. However, recent data showing CBS and CSE mRNA using quantitative PCR (polymerase chain reaction) suggest there are relatively equal amounts of both CBS and CSE in mouse brain tissue (Tyagi, et al. 2009a). Indeed, other publications have located CBS in the liver, kidney, pancreas (Bao, et al. 1998), lung (Kumaraswamy, et al. 2009), fibroblasts, smooth muscle cells (Wallace, et al. 2009) and data from this thesis has demonstrated CBS mRNA in human endothelial cells (Chapter 3). Therefore, CBS and CSE should not be thought of as two separate H_2S synthesising enzymes within separate body systems. Indeed, it is quite likely that both CSE and CBS work together in each organ system to generate H_2S (see 1.5.1. generation of H_2S from the transsulfuration pathway).

1.5.1. Generation of H_2S from the transsulfuration pathway

The generation of endogenous H_2S is thought to occur predominately during the trans-sulfuration pathway of L-homocysteine to L-cysteine. The transfer of sulfur between L-homocysteine and L-cysteine is described as trans-sulfuration, and is one of the means by which the toxic L-homocysteine is removed from cells (Persa, et al. 2004). CBS and

CSE are an integral part of the trans-sulfuration pathway which is interconnected with the transmethylation pathway between L-methionine and L-homocysteine (Figure 1.4.1) (Riedijk, et al. 2007). In these pathways, L-homocysteine is either catabolised to L-cysteine via the transulfuration pathway or remethylated by methionine synthase to methionine (Persa, et al. 2004). Both CSE and CBS can catalyse a number of reactions leading to the generation of H_2S as described in Table 1.4.1 and Table 1.4.2 respectively. CSE can catalyse a number of replacement or elimination reactions at the α, β or γ carbon in the substrates L-cysteine, L-homocysteine and L-cystathionine (Chiku, et al. 2009). In contrast, most of the replacement or elimination reactions related to CBS demonstrate a preference at the β-carbon on L-cysteine and L-homocysteine *in vitro* (Singh, et al. 2009). Interestingly, it has been demonstrated that CBS can contribute up to 68% of the H_2S produced whereas CSE contributes to the remaining 32%, on assumption of full enzyme activity and equal ratio of both enzymes (Table 1.4.3.) (Singh, et al. 2009). However, whether CBS is the predominant source of H_2S *in vivo* has not yet been explored. Indeed, the relative concentration for each enzyme and the presence of allosteric activators e.g. SAM may vary from tissue to tissue.

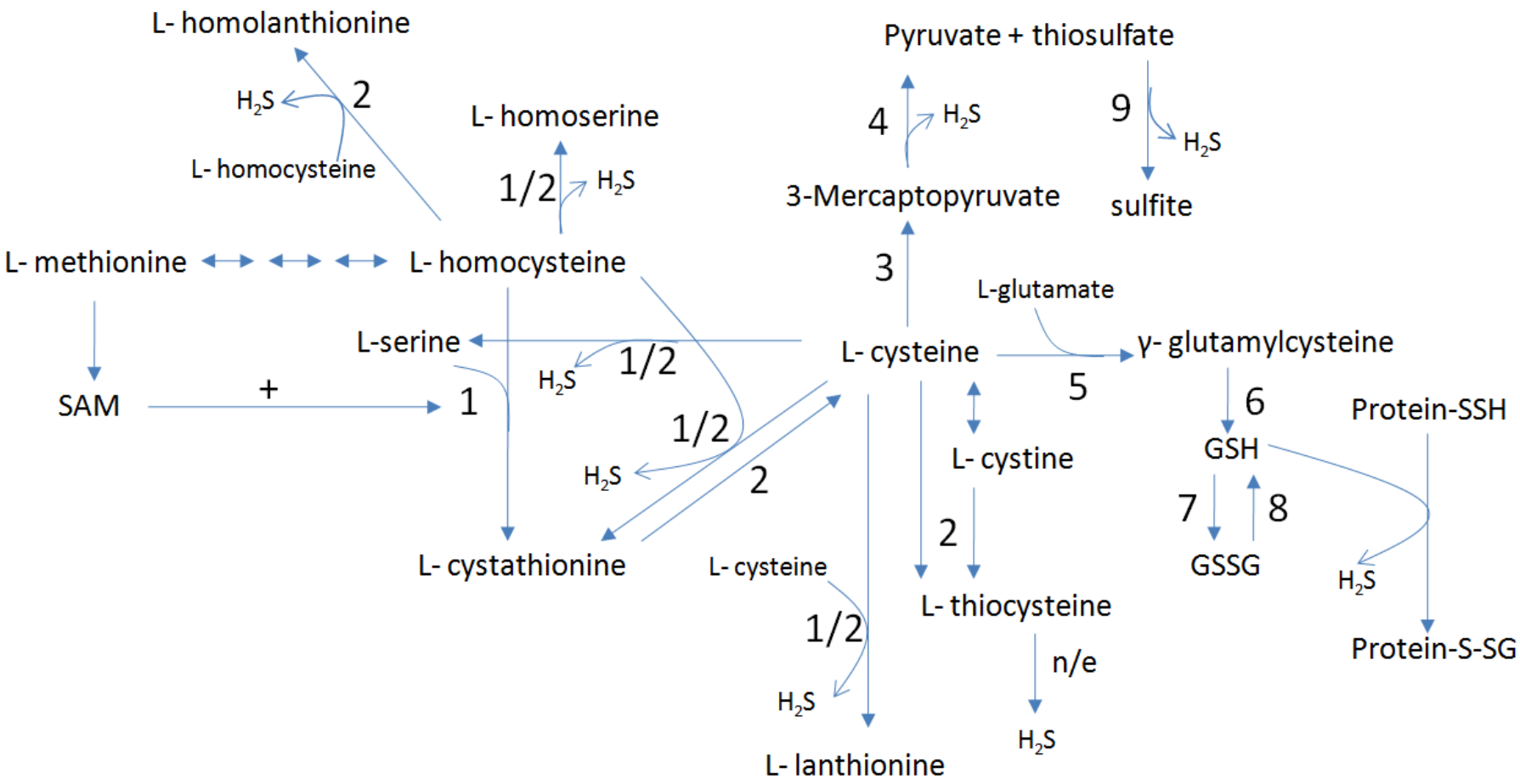

Figure 1.4.1. Endogenous generation of H_2S. 1 = CBS, 2 = CSE, 3 = cysteine aminotransferase (CAT), 4 = 3-mercaptopyruvate sulfurtransferase (3-MST), 5=gamma-glutamylcysteine synthase (rate limiting), 6 = Glutathione Synthase, 7 =Glutathione peroxidase, 8 = Glutathione reductase, 9 = Rhodanese, n/e = non-enzymatic decomposition, S-adenosylmethionine (SAM) (Kabil and Banerjee 2010; Iciek and Wlodek 2001; Persa, Pierce et al. 2004; Brosnan and Brosnan 2006; Chiku, Padovani et al. 2009; Singh, Padovani et al. 2009; Mikami, Shibuya et al. 2011)

Table 1.4.1. H_2S-generating reactions by CSE. Relative contribution (%) of H_2S produced in the presence of physiologically relevant concentrations of L-cysteine (100 µM) and L-homocysteine (10 µM) (Chiku, et al. 2009).

Reaction	Contribution to the production of H_2S (%)
a) L-cysteine + H_2O → L-serine + H_2S	70%
b) L-homocysteine + H_2O → L-homoserine + H_2S	29%
c) L-cysteine + L-cysteine → L-lanthionine + H_2S	Negligible
d) L-homocysteine + L-homocysteine → L-homolanthionine + H_2S	Negligible
e) L-homocysteine + L-cysteine → L-cystathionine + H_2S	Negligible

Table 1.4.2. H_2S-generating reactions by CBS. Relative contribution (%) of H_2S produced in the presence of physiologically relevant concentrations of L-cysteine (100 µM), L-homocysteine (10 µM) and S-adenosylmethionine (360 µM, SAM) (Singh, et al. 2009).

Reaction	Contribution to the production of H_2S (%)
a) L-cysteine + H_2O → L-serine + H_2S	2.6%
b) L-cysteine + L-cysteine → L-lanthionine + H_2S	1.6%
c) L-homocysteine + L-cysteine → L-cystathionine + H_2S	96%

Table 1.4.3. Percentage of H_2S-generated by CSE or CBS (ratio 1:1) in different conditions (Singh, et al. 2009).

Condition	CSE	CBS
a) L-cysteine (100 μM) + L-homocysteine (10 μM) + SAM (360 μM) (full activation of CBS)	32%	68%
b) L-cysteine (100 μM) + L-homocysteine (10 μM) (absence of SAM)	80%	20%
c) L-cysteine (100 μM) + L-homocysteine (40 μM) + SAM (360 μM) (moderate hyperhomocysteinemia)	45%	55%
d) L-cysteine (100 μM) + L-homocysteine (200 μM) + SAM (360 μM) (severe hyperhomocysteinemia)	74%	26%

1.5.2. Other pathways of endogenous H_2S generation

An alternative enzymatic pathway to the transulfuration pathway for the enzymatic generation of H_2S is the 3-mercaptopyruvate pathway of H_2S generation. This pathway involves two enzymes 3-mercaptopyruvate sulfurtransferase (3-MST, EC 2.8.1.2) and the P5'P dependent enzyme cysteine/aspartate transaminase (CAT, identical enzymes EC 2.6.1.3/ EC 2.6.1.1). Currently, the brain (Shibuya, et al. 2009b), rat vascular endothelium (Shibuya, et al. 2009a), and rat retinal neurons (Mikami, et al. 2011b) have been demonstrated to synthesise H_2S by this pathway. The subcellular location by which these enzymes produce H_2S is unclear. 3-MST is predominately located in the mitochondria, but is also found in the cell cytoplasm in lesser amounts (Kimura 2011). In this pathway, CAT catalyses the reaction between L-cysteine and α-ketoglutarate to produce 3-mercaptopyruvate and L-glutamate (Nagahara and Sawada 2006, Tanizawa 2011). 3-MST subsequently catalyses the reaction between 3-mercaptopyruvate and sulphurous acid (HSO_3^-) resulting in the production of pyruvate and either a thiosulfate (Mikami, et al. 2011a, Tanizawa 2011) or a persulfide (R-SSH) (Kabil and Banerjee 2010). The persulfide or thiosulfate is subsequently reduced (possibly by another sulftransferase) in the presence of reducing agents such as dihydrolipoic acid or thioredoxin to release H_2S (Mikami, et al. 2011a). The relative contribution of H_2S generation from the transulfuration pathway and 3-MST/CAT pathway is yet unknown and cannot be directly compared as the activities of 3-MST and CAT have been characterised at pH >9.5 and in rodents only (Shibuya, et al. 2009b, Kabil and Banerjee 2010). Interestingly, dithiothreitol is utilised in the methods to measure H_2S synthesising activity via the 3-MST/CAT pathway (Shibuya, et al. 2009a, Shibuya, et al.

2009b). Dithiothreitol is a strong reducing agent and is capable of inducing the release of H_2S from protein bound sulfur (Tangerman, et al. 2002). Whether protein desulfuration or the 3-MST/CAT pathway is the reason for the H_2S generated in the former study (Shibuya, et al. 2009b) is yet unclear.

Rhodanese (also known as thiosulfate sulfurtransferase, EC: 2.8.1.1), another mitochondrial enzyme with similar homology to 3-MST, is also capable of producing H_2S from thiosulfate (Mikami, et al. 2011a). In this reaction, rhodanese catalyses thiosulfate in the presence of a reducing agent such as dihydrolipoic acid to form sulfite (SO_3^{2-}) and H_2S (Koj, et al. 1967, Mikami, et al. 2011a). Interestingly, this enzyme has also been proposed to detoxify H_2S (Picton, et al. 2002), possibly by catalysing the transfer of sulfur from thiosulfate to thiol (-SH) compounds to produce persulfides (Schlesinger and Westley 1974). However, the role for rhodanese in H_2S detoxification has been met with some controversy (Wilson, et al. 2008). It has been suggested that rhodanese does not metabolise sulfide (Wilson, et al. 2008). Instead the reported ability of rhodanese to metabolise sulfide is due to the spontaneous conversion of sulfide to thiosulfate and/or contamination with a sulfide oxidase (Wilson, et al. 2008). Instead, rhodanese is thought to utilise thoisulfate, rather than sulfide, as a substrate (Wilson, et al. 2008).

Other miscellaneous sources of H_2S in the body include the blood where erythrocytes are also capable of producing H_2S by reducing elemental sulfur on oxidation of glucose (Searcy and Lee 1998). In the gut, bacterial flora are capable of reducing sulfate to H_2S (Macfarlane, et al. 1992).

1.6. Endogenous regulation of H_2S synthesising enzymes

1.6.1. Regulation of CBS

H_2S synthesising enzymes CSE and CBS are regulated by a number of mechanisms. SAM can allosterically activate CBS by binding to the C-terminal regulatory domain (Scott, et al. 2004). Furthermore, the haem in CBS is redox-active; and the oxidation of haem from Fe^{2+} to Fe^{3+} increases CBS activity (Taoka, et al. 1998). H_2S donors, NaHS and GYY4137 have been demonstrated to down regulate CBS mRNA in human airway smooth muscle cells (Perry, et al. 2011), suggesting H_2S itself can negatively regulate CBS expression.

Insulin has been shown to inhibit CBS expression at the mRNA level, which correlates with increased liver CBS activity in diabetic rats (Ratnam, et al. 2002). Glucocorticoids have also been reported to increase CBS mRNA in a rat hepatoma cell line, H4IIE (Ratnam, et al. 2002). In astrocytes, CBS protein was up-regulated by epidermal growth factor (EGF), transforming growth factor (TGF-α), 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate (CPT-cAMP) and the synthetic glucocorticoid dexamethasone (Enokido, et al. 2005).

Nuclear factor Y (NF-Y), specificity protein (SP)1, SP3, and upstream stimulatory factor 1 (USF-1) are thought to transactivate the CBS promoter (Ge, et al. 2001a). Myeloid zinc finger protein 1 (MZF1) has also been demonstrated to stimulate CBS promoter activity in HT1080 cells but not HepG2 cells (Ge, et al. 2001b).

1.6.2. Regulation of CSE

Compared to CBS, less is known about the regulation of CSE. Lipopolysaccharide (LPS) has been shown to stimulate CSE protein expression in RAW264.7 cells (Zhu, et al. 2010). Liver and kidney CSE activity and mRNA expression was shown to be up-regulated in LPS treated mice which may suggest CSE expression is regulated by NF-κB (Li, et al. 2005). CSE is regulated by Ca^{2+}/CaM, and endothelial cells produce H_2S in response to Ca^{2+} stimulating agents (Yang, et al. 2008). Similar to CBS, NaHS (50 – 500 μM) has been shown to reduce CSE mRNA expression in mouse aortic smooth muscle cells (Wang, et al. 2009b), suggesting H_2S exerts a negative feedback mechanism for CSE.

MZF1 and SP1 are thought to increase the basal transcriptional activity of CSE in HEK-293 cells (Ishii, et al. 2004). High concentrations of glucose (20 mM) have also been demonstrated to down-regulate CSE expression in isolated rat pancreatic islets as a result of p38 MAPK activation and SP1 phosphorylation (Zhang, et al. 2011). Acute myeloid leukaemia-1a (AML-1a), USF-1 and N-Myc are thought to suppress CSE transcriptional activity in HEK-293 cells (Ishii, et al. 2004). Another consensus sequence associated with CSE transactivation includes signal transducers and activators of transcription x (STATx) (Ishii, et al. 2004).

Interestingly, both CSE and CBS appear to be regulated by the common transcription factors SP1, MZF1 and USF-1, which may suggest these are the universal transcription factors in regulating the transulfration pathway.

1.6.3. Gasotransmitter-gasotransmitter regulation

Evidence from pharmacological compounds suggests that all three gasotransmitters, H_2S, $^{\bullet}NO$ and CO, are capable of interacting and regulating each other's activity. The H_2S donor, NaHS 100 μM, has been shown to attenuate LPS induced iNOS expression and nitrate/nitrite production in microglia (Hu, et al. 2007). NaHS (50 – 200 μM) has also been demonstrated to reduce nitrate/nitrite production and iNOS expression through up-regulating haem-oxygenase-1 (HO-1, the enzyme involved in CO biosynthesis) in LPS stimulated RAW264.7 cells (Oh, et al. 2006). Conversely, NaHS (100 μM) increases IL-1β induced iNOS protein expression in rat vascular smooth muscle cells (Jeong, et al. 2006). Moreover NaHS (100 μM) has been demonstrated to reduce eNOS phosphorylation in HUVEC (Geng, et al. 2007), and induce eNOS phosphorylation in cardiomyocytes (in a model of ischemia-reperfusion) (Yong, et al. 2008). H_2S has also been suggested to increase cGMP (cyclic 3',5'-guanosine monophosphate) levels by inhibiting phosphodiesterase activity *in vitro* (Bucci, et al. 2010). In a rat model of recurrent febrile seizures, NaHS was shown to up-regulate HO-1 in the rat brain (Han, et al. 2006). In a rat model of hemorrhagic shock, a bolus injection of NaHS (0.2 mg/kg i.v.) significantly increased HO-1 and HO-2 protein expression in heart and aortic tissue (Ganster, et al. 2010). Conversely, DL-propargylglycine (PAG, 2 mM), an irreversible inhibitor of CSE was shown to increase HO-1 expression in rat aortic smooth muscle cells; whereas NaHS (100 μM) decreased HO-1 expression in this cell line (Jin, et al. 2006).

The $^{\bullet}NO$ donor SNAP (*S*-nitroso-*N*-acetylpenicillamine, 100 μM) has also been demonstrated to increase CSE mRNA expression in cultured vascular smooth muscle cells (Zhao, et al. 2001). Moreover, $^{\bullet}NO$ donor, SNP (sodium nitroprusside, 1 μM) was

shown to significantly increase H_2S synthesising enzyme activity in rat fetal membranes (amnion) (Patel, et al. 2009) and increased H_2S production in rat aortic homogenates (Zhao, et al. 2001). $^{\bullet}NO$ and CO can also directly inhibit CBS activity by binding to the haem, with an inhibition constant of 320 ± 60 μM and 5.6 ± 1.9 μM respectively (Taoka, et al. 1999, Taoka and Banerjee 2001).

Apart from "gas-enzyme" interaction, "gas-gas" interaction has also been described. Both $^{\bullet}NO$ and H_2S have been shown to chemically react to form novel less biologically active nitrosothiol (RSNO) (Whiteman, et al. 2006). Incubation of SNP with NaHS in cultured RAW264.7 cells did not elevate cGMP levels, compared to SNP alone, unless $^{\bullet}NO$ was released in the presence of copper (Cu^{2+}, a reducing agent that cleaves the RS-NO bond) (Whiteman, et al. 2006). In anaesthetised rats SNP induced a significant reduction in mean arterial blood pressure, whereas co-administration of SNP and NaHS abolished this reduction in blood pressure (Ali, et al. 2006).

Taken as a whole, it appears that H_2S donors can both activate and inactivate NOS and HO. Currently there does not appear to be data in the literature demonstrating whether CO donors also regulate NOS or H_2S synthesising activity. Overall, this confusing evidence suggests that these gassotransmitters are capable of regulating each other's activity. Indeed, whether this interaction occurs endogenously is currently unknown and is an area which this thesis aims to explore.

1.7. **Insight into the endogenous functions of CSE and CBS from gene alteration**

1.7.1. CBS KO mice

$CBS^{-/-}$ knock out (KO) mice are typically used to model hyperhomocysteinemia. However, it is possible that $CBS^{-/-}$ KO mice will also have altered H_2S synthesising activity. $CBS^{-/-}$ mice have a typical phenotype of: normal birth weight with hindered growth and delayed eye opening compared to WT (wild type) mice (Watanabe, et al. 1995); hyperhomocysteinemia (total plasma homocysteine 271.1 ± 61.5 μM c.f. 7.4 ± 2.9 μM); elevated liver SAM (35.6 ± 5.9 nmol/g c.f. 19.1 ± 6.1 nmol/g) (Choumenkovitch, et al. 2002); hypermethioninemia (plasma methionine 2,640 ± 317 μM c.f. 117 ± 9 μM); a reduction in plasma taurine (165 ± 60 μM c.f. 617 ± 242 μM) (Ishii, et al. 2010). Very few of these mice survive past 5 weeks after birth (Watanabe, et al. 1995). Surviving $CBS^{-/-}$ mice display increased collagen deposition in the lung, alveolar septal thickening, and air space enlargement associated with increased TGF-β (transforming growth factor-β) mRNA (Hamelet, et al. 2007), suggesting a role for CBS in airway remodeling. The liver of $CBS^{-/-}$ mice have a light tan colour compared to reddish-brown colour of WT mice; and the hepatocytes are multinucleated, enlarged and contain fatty deposits (Watanabe, et al. 1995) which indicates a role for CBS in normal liver function.

$ApoE^{-/-}/CBS^{-/-}$ double KO mice have provided insight into the role of CBS in atherosclerosis (Wang, et al. 2003). Within 6 months, $ApoE^{-/-}/CBS^{-/-}$ fed on a normal diet had significantly ($P<0.05$) greater number of atherosclerotic lesions compared to $ApoE^{-/-}/CBS^{+/+}$. (Wang, et al. 2003) The increased risk of atherosclerotic lesions in the $ApoE^{-/-}/CBS^{-/-}$ were associated with an increased uptake of acetylated-LDL (low density

lipoprotein) by peritoneal macrophages suggesting an altered macrophage lipoprotein metabolism (Wang, et al. 2003).

$CBS^{-/+}$ heterozygote KO mice have previously been utilised to model mild hyperhomocysteinemia. These mice have normal growth but with twice the plasma homocysteine compared to WT (13.5 + 3.2 nmol/ml c.f. 6.1 ± 0.8 nmol/ml) (Watanabe, et al. 1995) and a reduction in plasma H_2S (~55 µM c.f. ~35 µM H_2S) (Sen, et al. 2009). $CBS^{-/+}$ mice have increased heart rate (HR, 520 ± 40 beats/min c.f. 435 ± 30 beats/min) and increased mean arterial blood pressure (MAP, 130 ± 1 mmHg c.f. 92 ± 1 mmHg) compared to WT (Ovechkin, et al. 2006). Moreover, $CBS^{-/+}$ mice also have impaired aortic relaxation in response to acetylcholine (ACh) as a result of endothelial cell dysfunction (Eberhardt, et al. 2000). In addition, $CBS^{-/+}$ mice have increased risk of thrombosis thought to result from hyperhomocysteinemia; as both $CBS^{+/+}$ and $CBS^{-/+}$ mice fed on a high methionine diet (to increase plasma homocysteine) have increased risk of thrombosis (Dayal, et al. 2006).

Overall, it appears that a deficiency in CBS results in cardiovascular dysfunction. Whether these effects are due to changes in plasma homocysteine or H_2S are currently unknown. Indeed, many of the studies investigating the effects of homocystinuria from CBS KO mice have not examined the consequences of changes in plasma H_2S levels. Therefore many of the effects described in those studies may possibly result from an alteration in H_2S generation.

1.7.2. CSE KO mice

In contrast to $CBS^{-/-}$ mice, $CSE^{-/-}$ mice have normal growth, fertility and survival (Yang, et al. 2008, Ishii, et al. 2010). These mice display significant ($P<0.05$): increases in plasma homocysteine (~18 µM c.f. 1 µM); reduction in plasma H_2S (~18 µM c.f. 40 µM); a reduction in plasma cysteine (~225 µM c.f. 300 µM) compared to WT (Yang, et al. 2008); these mice also display lower levels of plasma taurine (190 ± 60 µM c.f. 498 ± 88 µM) (Ishii, et al. 2010). Unlike $CBS^{-/-}$ mice, $CSE^{-/-}$ mice display cystathioninuria (elevated levels of cystathionine in the urine, 33.1 ± 5.3 mM c.f. 0.00) but not hypermethioninemia (elevated plasma methionine) (Ishii, et al. 2010) consistent with a predominant role for CBS in metabolising methionine and CSE for cystathionine. Unlike $CBS^{-/-}$ mice, $CSE^{-/-}$ are free of hepatic steatosis and show no biochemical signs of liver damage (no significant difference in aspartate and alanine aminotransferase levels compared to WT) (Ishii, et al. 2010). Similar to $CBS^{-/-}$ mice, restricting cysteine in the diet of $CSE^{-/-}$ mice led to weight loss, growth retardation, reduced glutathione production, muscular atrophy and death within 2 weeks; and injecting NaHS (39 µmol/kg i.p.) over 10 weeks, not surprisingly, did not reverse these effects (Ishii, et al. 2004, Mani, et al. 2011).

Several functions for CSE have recently been described. The effect of CSE on blood pressure regulation is controversial: one group has suggested $CSE^{-/-}$ mice develop aged dependent hypertension (~135 mmHg c.f. 117 mmHg) (Yang, et al. 2008); whereas $CSE^{-/-}$ mice from another group did not display altered effects on blood pressure compared to WT (Ishii, et al. 2010). Cultured smooth muscle cells isolated from the mesenteric arteries of $CSE^{-/-}$ have increased proliferation rate compared to

their WT counterparts (Yang, et al. 2010) and may be one reason for the hypertensive phenotype described in the former study (Yang, et al. 2008). Indeed, this effect on smooth muscle cell proliferation may suggest a role for CSE in vascular remodeling (Yang, et al. 2010). Microvessel formation induced by vascular endothelial growth factor (VEGF) was significantly reduced in the aortic rings isolated from $CSE^{-/-}$ mice compared to WT suggesting a role for CSE in angiogenesis (Papapetropoulos, et al. 2009). Endogenous CSE may play a role in O_2 sensing in the glomus cells of the carotid body (Peng, et al. 2010). In this study, $CSE^{-/-}$ mice were impaired of a ventilatory response to hypoxia, suggesting the impairment of an O_2 sensing system (Peng, et al. 2010).

CSE may have an endogenous role in cardiac tissue (Salloum, et al. 2009). The phosphodiesterase-5 inhibitor tadalafil (1 mg/kg i.p. pre-treatment) has been demonstrated to reduce myocardial infarct size and this drug effect was abolished in $CSE^{-/-}$ mice, suggesting a role for endogenous CSE in cardioprotection (Salloum, et al. 2009).

Endogenous CSE may also have a role to play in the development of diabetes as $CSE^{-/-}$ mice had a delayed onset in a model of streptozotocin (STZ)-induced diabetes (Yang, et al. 2011b).

Similar to $CBS^{-/-}$ mice, $CSE^{-/-}$ mice appear to have cardiovascular dysfunction. $CBS^{-/-}$ and $CSE^{-/-}$ mice have both an elevated plasma homocysteine and a reduction in plasma H_2S and may be a problem when using CSE/CBS KO mice.

1.7.3. Functions of CBS/CSE – evidence from genetic defects in humans

The incidence of humans displaying CBS deficiency may range from 1:20,000 to 1:1,000,000 (Naughten, et al. 1998, Gaustadnes, et al. 1999, Moat, et al. 2004). A deficiency of CBS in humans results in the hereditary multisystemic disorder hyperhomocysteinemia (Miles and Kraus 2004, Qu, et al. 2008). These patients also have an elevated plasma SAM, SAH and methionine (Finkelstein 2006). This autosomal recessive phenotype is characterised by: bone deformities, such as osteoporosis and scoliosis; effects on the central nervous system e.g. mental retardation and abnormalities in the electrocardiogram; increased risk of thrombosis and atherosclerosis formation (De Franchis, et al. 1998, Fonseca, et al. 1999). In contrast to KO mice studies, patients do not develop lung fibrosis (Maclean, et al. 2010). In humans, CBS is located on chromosome 21 and is expressed three times greater in the brains of Down's syndrome (or trisomey 21) patients than in control; and may be a cause of the cognitive dysfunction described in Down's patients (Ichinohe, et al. 2005).

In contrast to CBS, the incidence of CSE deficiency is much lower. These patients have increased cystathionine in the kidney, liver, brain and cerebrospinal fluid; and mild to moderate hyperhomocysteinemia (Finkelstein 2006). These patients have no obvious symptoms or abnormalities possibly due to a sufficient dietary intake of cysteine (Kraus, et al. 2009). Interestingly, no significant difference was found in a clinical study assessing the occurrence of single nucleotide polymorphisms in CSE of hypertensive patients (n = 503) and normotensive controls (n = 490) of the Northern Chinese Han population, which may suggest CSE is not associated with essential hypertension (Li, et al. 2008d). Due to low sample numbers in the latter study, further work is necessary to confirm this observation.

1.7.4. Homocysteine or H_2S?

CBS heterozygous KO mice ($CBS^{-/+}$) mice are commonly used as a model of homocysteinemia (Sen, et al. 2009). However, these mice also display a reduction in serum H_2S, in addition to elevated levels of homocysteine (Sen, et al. 2009). It is interesting to note that in a model of hyperhomocysteinemia, supplementing NaHS (30 µM) in the drinking of water of $CBS^{-/+}$ KO mice increased their plasma H_2S levels and led to a normalisation of urinary protein secretion indicating improved renal function (Sen, et al. 2009). The effects of improved renal function as a result of H_2S supplementation was thought to be a consequence of the prevention of apoptotic death in renal cortical tissues, reduced activity of matrix metalloproteinase-2 and -9 and a reduction in kidney superoxide levels (Sen, et al. 2009). These authors suggested that hyperhomocysteinemia-associated renal damage is due to a reduction in endogenous H_2S levels (Sen, et al. 2009).

H_2S may be an endogenous regulator of blood pressure (Yang, et al. 2008). Interestingly there is no correlation between homocysteine and blood pressure in humans (Sundstrom, et al. 2003). In a rat model of hyperhomocysteinemia, induced by a high methionine diet, an increased MAP was associated with elevated levels of homocysteine and a reduction in plasma H_2S (Sowmya, et al. 2010). These authors suggested H_2S may regulate blood pressure in hyperhomocysteinemia (Sowmya, et al. 2010). However, in another model, $CBS^{-/+}$ mice fed 3-deazaadenosine (to inhibit the accumulation of homocysteine) resulted in the normalisation of MAP and HR (93 ± 1 mmHg and 469 ± 27 beats/min) (Ovechkin, et al. 2006), which may suggest homocysteine accumulation is the cause of the hypertension. Conversely, increasing

homocysteine levels in WT mice by increasing their methionine consumption/intake for >6 weeks did not affect blood pressure suggesting that homocysteine is not the cause of the rise in blood pressure seen in the $CSE^{-/-}$ mice (Yang, et al. 2008). Instead, the latter study suggested that the reduction in plasma H_2S was the cause of the increase in blood pressure described in the $CSE^{-/-}$ mice (Yang, et al. 2008). Indeed, whether H_2S and/or homocysteine, regulates blood pressure in rodents is yet unclear.

The injection of H_2S in a rat model of hyperhomocysteinemia induced by subcutaneous injection with homocysteine resulted in a reduction in total plasma homocysteine (Chang, et al. 2008). Moreover, the oxidative stressed induced by homocysteine in the myocardium was associated with the down regulation of CSE, and the oxidative stress induced in the myocardium was alleviated upon injection of exogenous H_2S (Chang, et al. 2008). This suggests that endogenous H_2S may have a role in the pathology of hyperhomocysteinemia.

Overall, it is likely that some of the physiological responses described in CSE/CBS KO mice are caused by removal of H_2S from the system. Whether the cardiovascular dysfunction seen with removal of CSE or CBS is as a result of H_2S and/or homocysteine is an area that requires further investigation. Indeed, many of the previous studies utilising CBS KO mice have not examined the effects of H_2S.

1.8. H_2S donors and CSE/CBS inhibitors

H_2S donating salts and CSE/CBS inhibitors have predominately been used to decipher the biological actions of H_2S. Although these tools have been of some value, it should be noted that both H_2S donors and inhibitors have their disadvantages. H_2S donors are capable of inducing cell death in cell types such as: hepatocytes (NaHS 500 μM, 60

min) (Eghbal, et al. 2004), pancreatic acini (NaHS 10 μM, 3 h) (Cao, et al. 2006), aortic smooth muscle cells (H_2S 200 μM, 2 h) (Yang, et al. 2004), and INS-1E cells (insulin-secreting β-cell line, H_2S 50 μM, 12 h) (Yang, et al. 2007). However, the role for H_2S donors in inducing apoptosis is controversial as this gas has also been shown to inhibit apoptosis or preserve cell viability: cardiac myocytes (in a model of myocardial ischemia/reperfusion, NaHS 3 mg/kg pretreatment) (Sivarajah, et al. 2009), SH-SY5Y (human neuroblastoma cell line, rotenone induced apoptosis, NaHS 1 – 300 μM, 24 h) (Hu, et al. 2009), hepatocytes (hepatic ischemia/reperfusion model, Na_2S 0.3 – 1 mg/kg pretreatment) (Jha, et al. 2008), retinal ganglion cells (model of ischemia/reperfusion, H_2S inhalation 80 ppm pretreatment) (Biermann, et al. 2011), and HCT116 cells (human colon cancer cells, β-phenylethyl isothiocyanate induced apoptosis, NaHS 0.1 – 1 mM) (Rose, et al. 2005). Indeed, although the role for H_2S donors in apoptosis has met with controversy, the potential for H_2S donors to induce apoptosis is a possibility and is a disadvantage of using H_2S salts as a tool to determine the biological actions of H_2S.

Many of the H_2S synthesising enzyme inhibitors are thought to be 'selective' for either CSE or CBS. However, most of these compounds inhibit other P5'P dependent enzymes. DL-Propargylglycine (PAG), irreversible inhibitor of CSE, is capable of inhibiting enzymes such as aspartate aminotransferase (Tanase and Morino 1976), alanine transaminase, (Burnett, et al. 1980, Cornell, et al. 1984) and methionine gamma-lyase (Lockwood and Coombs 1991). Aminooxyacetate acid (AOAA), a reversible inhibitor of CBS, is capable of inhibiting aspartate aminotransferase (Rej 1977), GABA aminotransferase (Loscher 1981), glutamate decarboxylase (Hamel, et al. 1982), glutamate transaminase (Seglen and Solheim 1978) and alanine aminotransferase (Smith, et al. 1977). Hydroxylamine (HA, inhibitor of CBS) has been

reported to inhibit ornithine aminotransferase, aspartate aminotransferase (Kito, et al. 1978) and alcohol dehydrogenase (Kaplan and Ciotti 1953). The non-selective effects of these inhibitors should be kept in mind when analysing the effects of inhibiting endogenous H_2S synthesis.

1.9. Biological roles for H_2S – evidence from H_2S donors and CSE/CBS inhibitors

Although H_2S donors and H_2S synthesising enzyme inhibitors have their disadvantages, these tools have provided much insight into what is known about the biological actions of H_2S to date.

1.9.1. H_2S and the nervous system

In the brain, H_2S biosynthesis is predominantly thought to be generated through the activation of CBS and there is evidence to suggest that H_2S may act as a neuromodulator (Abe and Kimura 1996). Exogenous H_2S has been demonstrated to facilitate long term potentiation via activation of NMDA receptors through increasing cAMP, suggesting H_2S may be involved in memory formation (Abe and Kimura 1996, Kimura 2000). H_2S has also been shown to induce Ca^{2+} waves in cultured astrocytes and hippocampus brain slices through the activation of Ca^{2+} channels, and was suggested to be a mechanism by which H_2S induces signals between neurons (Nagai, et al. 2004).

NaHS (100 - 500 μM) has also been demonstrated to increase intracellular Ca^{2+} in cultured microglia cells, the resident macrophages in the CNS, partially via an adenylyl cyclase dependent mechanism (Lee, et al. 2006). This suggests that H_2S may play a role in neuroinflammation through microglia cell activation (Lee, et al. 2006). Interestingly, microglia cells pre-treated with NaHS (100 – 300 μM) caused a significant

reduction in LPS-induced nitrite production through inhibiting p38 MAPK phosphorylation (Hu, et al. 2007). Similarly, pre-treatment with SAM (100 µM, allosteric activator of CBS) significantly reduced LPS-induced nitrite production in microglia cells suggesting both exogenous and endogenous H_2S is anti-inflammatory (Hu, et al. 2007).

H_2S has been shown to protect neurons from oxidative stress induced by glutamate *in vitro* by up-regulating cysteine transport and increasing γ-glutamylcysteine synthetase activity and thus GSH production (Kimura and Kimura 2004). Moreover, NaHS (50 µM pretreatment) has been shown to reduce NOX-4 expression and reduce intracellular ROS production induced by methionine in bEnd3 cells, a mouse brain endothelial cell line (Tyagi, et al. 2009b).

In a rat model of recurrent febrile seizures induced by hyperthermia, NaHS (56 µmol/kg, i.p.) significantly increased γ-aminobutyric acid (GABA) B receptor subunits back to control levels (Han, et al. 2005b). This suggests that H_2S is capable of regulating synaptic excitation/inhibition through regulating GABA subunit expression in the brain in this model of febrile seizure (Han, et al. 2005b).

Mental retardation is a frequent symptom in patients with CBS deficiency (Dutta, et al. 2005), which may suggest CBS and/or endogenous H_2S production in the brain have roles in conscious intellectual activity. In humans, CBS is expressed on choromosome 21 (Belardinelli, et al. 2001). Trisomy 21 (Down syndrome) patients are often associated with mental retardation and have greater urinary thiosulfate levels compared to control possibly as a result of increased H_2S production (Belardinelli, et al. 2001). These controversial outcomes may suggest that CBS/H_2S may be an independent factor to conscious intellectual activity.

The hypothalamus has a number of brain functions including the regulation of the body temperature, hormone secretion, food intake and control of the autonomic nervous system. The hypothalamus is capable of secreting hypothalamic-releasing hormones such as corticotropin-releasing hormone (CRH) and growth hormone-releasing hormone (GHRH), which in turn influences the release of hormones from the pituitary gland. One study has demonstrated that H_2S may be capable of modulating the hypothalamo-pituitary (HPA) axis. NaHS (1 mM) has been demonstrated to significantly inhibit the release of CRH from *ex vivo* hypothalamus explants induced by KCl (Dello Russo, et al. 2000). However, NaHS did not have effect on CRH release in the unstimulated hypothalamus (Dello Russo, et al. 2000). Indeed, whether physiological concentrations of H_2S are capable of modulating the HPA axis is unclear and remains to be explored.

Transient receptor potential (TRP) A1 ion channel (TRPA1) is an ion channel found on capsaicin-sensitive sensory neurons of the peripheral nervous system (Streng, et al. 2008). It was initially characterised as a noxious cold receptor but is now thought to be a channel involved in mechanosensation (Streng, et al. 2008). NaHS has been demonstrated to activate TRPA1 ion channels on unmyelinated C-fiber afferents of the rat urinary bladder (Streng, et al. 2008) and may be a mechanism by which H_2S can initiate micturition. Furthermore, NaHS increases intracellular Ca^{2+} in TRPA1-expressing Chinese hamster ovary (CHO) cells (Streng, et al. 2008). This evidence suggests that H_2S may function as a nociceptive messenger by sensitising T-type Ca^{2+} channels and possibly TRPA1 channels on peripheral nerve fibers (Kawabata, et al. 2007).

1.9.2. H_2S and the pancreas

There appears to be increasing interest in H_2S and the regulation of endocrine function, particularly in the pancreas. H_2S has previously been demonstrated to activate K_{ATP} channels and induce hyperpolarisation in smooth muscle cells (Zhao, et al. 2001). Interestingly, the release of insulin is dependent on the inactivation of the K_{ATP} channels found on the β-islets of Langerhans. The presence of glucose results in the rise of intracellular ATP concentrations which leads to K_{ATP} channel inactivation, resulting in membrane depolarisation and opening of voltage gated Ca^{2+} channels and finally insulin release (Huopio, et al. 2002). In the presence of ADP (or less ATP), K_{ATP} channels open, resulting in membrane hyperpolarsation leading to closure of Ca^{2+} channels and suppression of insulin secretion (Huopio, et al. 2002). Both endogenous and exogenous H_2S has also been shown to increase the open probability of single-channel K_{ATP} channels in INS-1E cells, an insulin-secreting cell line (Yang, et al. 2005). Interestingly, the INS-1E cell line appears to be susceptible to apoptosis induced by H_2S (100 μM), possibly through activation of P38 MAPK pathway and endoplasmic reticulum stress (Yang, et al. 2007). Indeed the loss of β-islets is thought to be one of the causes of diabetes (Butler, et al. 2003).

In a rat model of streptozotocin-induced diabetes, an increase in H_2S enzyme activity in liver and pancreas was noted, and this was associated with an increase in CBS mRNA in both liver and pancreas (Yusuf, et al. 2005). The increased enzyme activity was normalised by insulin administration into the diabetic rats (Yusuf, et al. 2005). In the zucker diabetic fatty rat model, the pancreas of these rats had increased CSE expression and increased H_2S production compared to control (Wu, et al. 2009). Moreover, zucker diabetic fatty rats injected with PAG (33.9 mg/kg i.p.) over 4 weeks,

lead to a significantly increased serum insulin levels (Wu, et al. 2009). These authors suggested that insulin release was impaired in zucker diabetic fatty rats as a result of increased H_2S production in the pancreas (Wu, et al. 2009). Interestingly, in a model of non-obese diabetic mice, the reduction in plasma H_2S was suggested to be the cause of vascular dysfunction in these animals (Brancaleone, et al. 2008). Similarly, type-2 diabetic and overweight humans have also been demonstrated to have reduced plasma H_2S levels compared to lean controls (Whiteman, et al. 2010a).

Indeed H_2S appears to play a role in regulating insulin release and may possibly play a role in the pathological consequences of vascular dysfunction in diabetes. However, the roles of H_2S in this field are still in its infancy and require further exploration.

1.9.3. H_2S and the gastrointestinal tract

H_2S is present in the gastrointestinal tract (GIT) with reported levels of up to 1 mM H_2S present in the cecum (Deplancke, et al. 2003). This is thought to be a consequence of sulfate-reducing bacteria (Deplancke, et al. 2003). Interestingly, sulfate supplementation into the drinking water (16.7 mM) over 7 days to a year, did not alter H_2S concentrations in the GIT but did alter the bacterial community profiles (Deplancke, et al. 2003). Although supplementary H_2S does not increase H_2S in the GIT, supplementing dietary sulfur amino acids has been shown to increase human fecal H_2S concentrations (Magee, et al. 2000). CSE and CBS enzymes are present throughout the GIT (Martin, et al. 2009), and may also contribute to the H_2S in the GIT.

Using immunohistochemistry both CSE and CBS has been demonstrated to be present in the rat colon (Wallace, et al. 2009). CBS was found to be predominately in

the fibroblast and smooth muscle cells (Wallace, et al. 2009). Conversely, CSE expression was more diffuse and was shown to be located in blood vessels, epithelial, crypt and goblet cells of the rat colon (Wallace, et al. 2009). The presence of these enzymes in the GIT may suggest that H_2S may play a role in GIT movement. Indeed, NaHS (EC_{50} 62.6 ± 4.7 µM) has been shown to relax the guinea pig ileum (Teague, et al. 2002). Furthermore, inhibiting CSE using PAG (1 mM) was shown to increase the contractile response of the guinea pig ilieum to field stimulation (Teague, et al. 2002). The predominant expression of CBS in the smooth muscle cell layer of the intestine (Wallace, et al. 2009) suggests that CBS has a greater role in gastric mobility than CSE.

There appears to be increasing interest in the therapeutic benefit of H_2S-releasing NSAIDS (non-steroidal anti-inflammatory drugs) in the GIT. In the presence of biological samples the ester bond linking the NSAID to the H_2S releasing moiety ADT-OH (5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione) is thought to be hydrolysed liberating the NSAID and ADT-OH which releases H_2S (Distrutti, et al. 2006b, Chattopadhyay, et al. 2011). Interestingly, these compounds have been demonstrated to be more potent at inhibiting colitis (Fiorucci, et al. 2007), ulcer healing (Wallace, et al. 2007b) and other models of gastric injury (Fiorucci, et al. 2005a) than NSAIDS without the H_2S releasing moiety. Moreover, in a mouse model of ethanol-induced gastric damage, NaHS (75 µmol/kg, p.o., 30 min pretreatment) prevented macroscopic gastric lesions and PAG (50 mg/kg, p.o., 30 min pretreatment) inhibited the protection induced by L-cysteine (Medeiros, et al. 2009). It has also been demonstrated that injecting PAG (50 mg/kg, i.p., twice daily) after the induction of colitis with trinitrobenzene sulfonic acid significantly ($P<0.05$) increased the mortality of these rats (Wallace, et al. 2009). Interestingly, injecting PAG in normal animals without colitis had

no effect on survival rate after 14 days (Wallace, et al. 2009). Moreover, 4 day post-treatment with NaHS (30 μmol/kg, intracolonical, twice daily) significantly ($P<0.05$) reduced the severity of the colitis, as determined by a reduction in TNF-α expression, colon thickness and damage score (Wallace, et al. 2009). H_2S-releasing NSAIDS appear to be a promising field of H_2S therapeutics. Indeed, ATB-429 (H_2S releasing mesalamine) is currently entering first stage clinical trials for inflammatory bowel disease (Childers, et al. 2008). However, in July 2010, $^{\bullet}NO$-releasing NSAID naproxcinod was rejected by the Food and Drug Administration for its indication in osteoarthritis, due to limited data on the long term safety effects of this drug in the cardiovascular and GIT (Song 2010). This suggests, although H_2S-NSAIDs may be beneficial in preclinical models of inflammatory disease, it is likely that there may be some time before these drugs will be available for patients.

1.9.4. H_2S and the respiratory system

H_2S appears to have a role in the respiratory system. In humans, there are reduced serum levels of H_2S in patients with asthma and this appears to correlate with disease severity (Wu, et al. 2008). H_2S donors, GYY4137 and NaHS (both 100 μM) have been demonstrated to inhibit human airway smooth muscle cell proliferation induced by FCS and IL-1β *in vitro,* and this effect was inhibited by CBS inhibitor CHH (O-(Carboxymethyl)-hydroxylamine hemihydrochloride), but not CSE inhibitor PAG (Perry, et al. 2011). Moreover, in the same study, both GYY4137 and NaHS reduced CBS, but not CSE, mRNA expression suggesting CBS is the predominant enzyme involved in producing H_2S in human airway smooth muscle cells (Perry, et al. 2011). Interestingly, PAG appeared to aggravate airway responsiveness in a rat cigarette smoke model,

suggesting endogenous CSE is involved in the bronchodilation in this model (Chen, et al. 2010). It is possible there may be differences in the roles for CSE/CBS between humans and rodents and/or differing roles between CSE and CBS in the respiratory system. In a model of ovalbumin induced asthma in the rat, administration of NaHS (14 µmol/kg i.p. pre-treatment) significantly reduced iNOS expression in lung tissue, inflammatory cell infiltration, airway mucus secretion, subepithelial fibrosis and goblet cell hyperplasia; suggesting exogenous H_2S is anti-inflammatory (Chen, et al. 2009). In contrast, in another study, H_2S was shown to irritate the airways. In this study, NaHS (50 mM, 200 μl, intratracheal) was shown to increase total lung resistance in anesthetised guinea pigs, and this effect was inhibited by tachykinin antagonists (Trevisani, et al. 2005). Moreover, NaHS (10 µM – 30 mM) concentration-dependently induced guinea-pig bronchial contraction *ex vivo* (Trevisani, et al. 2005). These authors concluded that exogenous H_2S induces neurogenic inflammatory responses in guinea-pig airways (Trevisani, et al. 2005).

A reduction of H_2S synthesising activity in the lung was described in an *in vivo* rat model of hypoxic vasoconstriction (Wei, et al. 2008). The administration of NaHS (14 µmol/kg i.p.) significantly reduced the increase of oxidised glutathione (GSSH) suggesting H_2S may acts as an antioxidant during oxidative stress in the lung (Wei, et al. 2008).

Acute lung injury is a potential cause of acute respiratory failure in the clinic (Li, et al. 2008c). In a model of acute lung injury induced by oleic acid, rats pre-treated with NaHS (3.1 mg/kg, i.p.) had increased partial pressure of oxygen in arterial blood, reduced pulmonary wet/dry weight and a reduction in polymorphonuclear cell infiltration (Li, et al. 2008c). Moreover, in the same model, H_2S reduced pro-

inflammatory cytokine IL-6 and chemokine IL-8 and increased anti-inflammatory cytokine IL-10 in the lung and plasma (Li, et al. 2008c).

To date, the role of H_2S in airway inflammation is unclear but appears to trend towards an anti-inflammatory role. Whether H_2S therapy will translate to the clinic is yet uncertain. Indeed, H_2S has a pungent smell and is unlikely to be patient compliant even if H_2S did exert beneficial effects in the respiratory system.

1.9.5. H_2S and the cardiovascular system

a) H_2S and blood pressure

A biological role for H_2S in the cardiovascular system was first described in 2001 (Zhao, et al. 2001). In this study, a bolus injection of NaHS (2.5 µmol/kg i.v.) caused a transient (30 sec) reduction in mean arterial blood pressure (MAP) of approximately 12.5 mmHg in anaesthetised rats (Zhao, et al. 2001). This vasoactive property of NaHS *in vivo* was mimicked by the K_{ATP} channel opener pinacidil (2.8 µmol/kg) and inhibited by pre-treatment with a K_{ATP} channel blocker glibenclamide (2.8 µmol/kg) (Zhao, et al. 2001). Moreover, NaHS induced a dose dependent relaxation (10 µM – 1 mM, IC_{50} = 125 µM) in phenylephrine pre-contracted rat aortic rings (Zhao, et al. 2001). The effect in *ex vivo* blood vessels studies was inhibited by glibenclamide (IC_{50} = 140 µM), but not by 4-aminopyridine (2.5 mM, voltage-dependent K^+ (Kv) channel blocker), indomethacin (10 µM, non-selective cyclooxygenase inhibitor), SQ22536 (100 µM, adenylate cyclase inhibitor), ODQ (10 µM, guanylate cyclase inhibitor), staurosporine (30 nM, protein kinase C (PKC) inhibitor), superoxide dismutase (SOD, 160 U/ml) or catalase (1000 U/ml) (Zhao, et al. 2001). This suggests that the vasorelaxant effects of

H_2S are due to activation of K_{ATP} channels, and not voltage-dependent K^+ channels, prostaglandins, cAMP, cGMP, PKC, $O_2^{\bullet-}$ or H_2O_2.

Currently the major mechanism underlying the vasoactive effect of H_2S is thought to be a direct action of H_2S to open K_{ATP} channels in vascular smooth muscle cells, causing the muscle cells to hyperpolarise and relax (Zhao, et al. 2001). This effect is a direct action of H_2S on K_{ATP} channels rather than an effect of H_2S on ATP metabolism due to three observations: (i) intracellular ATP concentration was fixed, (ii) when H_2S were washed out, the effect on K_{ATP} channels were promptly reversed and, (iii) varying intracellular ATP concentrations did not change the effect of H_2S on K_{ATP} channels (Zhao, et al. 2001).

It has also been demonstrated that Cl^-/HCO_3^- channel inhibitor DIDS (1 mM) can also abolish H_2S induced relaxation in the rat aortic rings (Kiss, et al. 2008). In the same study, H_2S was shown to reduce ATP levels in rat aortic rings (Kiss, et al. 2008). Consequently, these authors hypothesised that the incubation of rat aortic rings with H_2S led to the inhibition of mitochondrial cytochorome c oxidase, reducing ATP generation, leading to cellular acidosis and the eventual activation of Cl^-/HCO_3^- channels to bring about vasodilatation (Kiss, et al. 2008).

Interestingly, the effect of H_2S on blood vessels were partially inhibited by the application of L-NAME (non-selective NOS inhibitor, 100 µM), charybdotoxin/apamin (inhibitors of endothelium-derived hyperpolarising factor (EDHF), both 50 µM) or removal of the endothelium; which may suggest H_2S stimulates the release of $^{\bullet}NO$ and/or EDHF from the endothelium, or H_2S itself may act as an EDHF (Zhao, et al. 2001, Zhao and Wang 2002). In contrast to the results of previous studies (Zhao, et al. 2001, Zhao and Wang 2002), it was recently reported that NaHS can increase cGMP levels in

rat aortic smooth muscle cells through inhibiting phosphodiesterase (PDE) activity, and may be an alternative method of inducing vasodilatation (Bucci, et al. 2010). Interestingly, more recently, H_2S has been demonstrated to enhance PDE activity in As4.1 juxtaglomerular cells (Lu, et al. 2011). Whether H_2S exerts isoform differences on PDE activity, is currently unknown.

Since 2001, H_2S has been shown to cause vasodilation in various vessels such as: the rat aorta, H_2S IC_{50}, 125 ± 14 μM (Hosoki, et al. 1997, Zhao, et al. 2001), rat mesenteric arteries, H_2S EC_{50} 25.2 ± 3.6 μM and NaHS EC_{50} 103.7 ± 10.0 μM (Cheng, et al. 2004), rabbit and human corpus cavernosum NaHS >100 μM (Srilatha, et al. 2007, d'Emmanuele di Villa Bianca, et al. 2009), human mammary artery NaHS >150 μM (Webb, et al. 2008), pig cerebral NaHS >10 μM (Leffler, et al. 2011), rat hepatic NaHS 100 μM (Fiorucci, et al. 2005b), and renal NaHS >10 nmol/min/kg (Xia, et al. 2009) vascular beds. Interestingly, NaHS 1 mM does not affect coronary flow rate (Geng, et al. 2004, Johansen, et al. 2006).

Although acute exposure to NaHS induces a transient (30 sec) reduction in blood pressure (Zhao, et al. 2001), H_2S may have longer lasting effects on blood pressure unrelated to vasodilatation. Chronic administration of NaHS has also been demonstrated to reduce blood pressure in spontaneously hypertensive rats possibly by inhibiting collagen deposition and ROS production (Shi, et al. 2007, Zhao, et al. 2008). Moreover, chronic administration of NaHS has also been demonstrated to reduce the blood pressure of rats induced with renovascular hypertension but not in normal control rats (Lu, et al. 2010). This effect was suggested to result from reducing renin release and synthesis in the kidney (Lu, et al. 2010), a mechanism suggested to be through inhibition of adenylate cyclase activity (Lu, et al. 2011).

Although exogenous H_2S has been demonstrated to induce vasodilation, the endogenous role of H_2S in blood pressure regulation is somewhat controversial. In one study, two daily injections of the irreversible CSE inhibitor PAG (with a total daily dose of 33.9 mg/kg i.p.) over 5 weeks caused a significant increase in systolic blood pressure within 2-3 weeks (Zhao, et al. 2003). However, in another study, a single injection of PAG (50 mg/kg, i.v.) had no effect on MAP or heart rate (HR) over 6 h in anaesthetised rats (Collin, et al. 2005). This is in contrast to L-NAME (1 – 10 mg/kg i.v.), which increases arterial blood pressure within 10 min (Gardiner, et al. 1990). $CSE^{-/-}$ KO mice have been described to develop an age dependent hypertension (Yang, et al. 2008). However, another group did not demonstrate blood pressure differences in their $CSE^{-/-}$ KO mice compared to WT (Ishii, et al. 2010).

Although H_2S is better known for its vasodilator effect, this gas can also cause vasoconstriction (e.g. alligator aorta, rat thoracic aorta) or a complex multiphasic vasoconstrictor and -dilator effect (e.g. duck and rat pulmonary artery), depending on the vascular bed and across different species (Dombkowski, et al. 2005). The mechanisms underlying its vasoconstrictive effects are unclear but several mechanisms have been proposed. It has been reported that H_2S is able to contract rat aortic rings at low NaHS concentrations (<100 µM, below the threshold of K_{ATP} channel activation), possibly by quenching concurrently released $^{\bullet}NO$ (Ali, et al. 2006), to form a novel nitrosothio (Whiteman, et al. 2006). NaHS (300 µM) has also been demonstrated to directly inhibit the conversion of [^{3}H]-arginine into [^{3}H]-citrulline by recombinant eNOS *in vitro* (Kubo, et al. 2007). However, whether H_2S can directly inhibit eNOS activity *in vivo* is yet unclear. In addition, H_2S can stimulate anion transporters to exchange HCO_3^- for $O_2^{\bullet-}$ which directly inactivates $^{\bullet}NO$ (Liu and Bian 2011). NaHS (5 µM) has also been

demonstrated to inhibit forskolin-induced cAMP accumulation in rat aortic vascular smooth muscle cells and was suggested to be another mechanism by which H_2S mediates its vasoconstrictive effects (Lim, et al. 2008) a mechanism possibly through stimulating phosphodiesterase activity and suppressing adenylate cyclase activity (Lu, et al. 2011). On the contrary, others have demonstrated H_2S can inhibit phosphodiesterase activity *in vitro* (Bucci, et al. 2010). Indeed, this is an area which requires further clarification.

Interestingly, hypoxia also induces vasodilation or vasoconstriction similar to that of H_2S (Olson, et al. 2006). It has been hypothesised that H_2S may serve as an O_2 sensor in vascular smooth muscle; where the balance of available H_2S (produced endogenously) is dependent on its oxidation by available O_2 (Olson, et al. 2006). Accordingly, when the O_2 drops (e.g. in hypoxic conditions), the concentration of H_2S increases which directly induces vasoconstriction/vasodilatation, or what is seen as hypoxic vasoconstriction/vasodilatation (Olson, et al. 2006). In accord with this hypothesis, a recent study demonstrated that in hypoxic conditions H_2S synthesising activity is increased in the rat carotid body (Peng, et al. 2010). Moreover, injecting rats with PAG (200 mg/kg) significantly reduced response of the carotid body to hypoxia, suggesting a role for H_2S in O_2 sensing (Peng, et al. 2010).

b) H_2S and cardiovascular remodeling

H_2S appears to play a role in vascular remodeling. Administration of NaHS (56 µmol/kg/day) over 5 weeks inhibited collagen deposition in the aorta of spontaneously hypertensive rats (Zhao, et al. 2008). Moreover, NaHS (50 µM) was shown to inhibit angiotensin II induced vascular smooth muscle cell proliferation *in vitro* (Zhao, et al.

2008). Daily injection of NaHS (30 μmol/kg i.p.) over 4 weeks significantly reduced neointima hyperplasia after balloon injury in rats; and this effect was associated with a reduction in vascular smooth muscle cell proliferation (Meng, et al. 2007).

$ApoE^{-/-}$ mice on a high fat diet injected with NaHS (56 μmol/kg) or PAG (37.5 mg/kg) daily over 10 weeks led to a significant reduction or enlargement of aortic atherosclerotic plaque formation respectively (Wang, et al. 2009b). The effect of NaHS in $ApoE^{-/-}$ was associated with a reduction in plasma and aortic ICAM-1 expression (Wang, et al. 2009b). In the same study NaHS (50 – 500 μM) inhibited the transformation of THP-1-derived macrophages to foam cell phenotype in the presence of oxidised LDL (Wang, et al. 2009b). NaHS (750 μM) has previously been demonstrated to inhibit the modification of LDL (1 mg/ml) induced by HOCl (1 mM) (Laggner, et al. 2007). Moreover, NaHS (75 – 300 μM) was shown to concentration dependently induce the decomposition hydrogen peroxide (H_2O_2) and scavenge hypochlorous acid (HOCl), components that can oxidise LDL (Laggner, et al. 2007). Indeed, others have also shown that H_2S can scavenge other reactive species including peroxynitrite ($ONOO^-$) (Whiteman, et al. 2004) and superoxide ($O_2^{\bullet-}$) (Chang, et al. 2008) all of which are capable of modifying LDL (Steinbrecher 1988, Dinis, et al. 2002).

Vascular calcification is a process in which VSMCs are transformed into a phenotype similar to that of osteoblasts; characterised by over expression of proteins such as osteopontin and increased alkaline phosphatase activity (Wu, et al. 2006). In a rat model of vascular calcification induced by vitamin D3/nicotine, injection of NaHS (2.8 μmol/kg) over 4 weeks caused a significant reduction in vascular calcium content, reduced alkaline phosphatase activity and a reduction in osteopontin mRNA (Wu, et al. 2006). Similarly, another study also demonstrated that NaHS (50 μM) could inhibit the

calcification of human aortic smooth muscle cells *in vitro* induced by inorganic phosphate (Zavaczki, et al. 2011). In the same study PAG (10 mM) significantly increased human aortic smooth muscle cell calcification (Zavaczki, et al. 2011).

In a rat model of heart failure, daily treatment with NaHS (3.136 mg/kg) improved left ventricular systolic pressure (LVSP) with no change in heart rate (Wang, et al. 2011b). Moreover, the rats injected with H_2S had significantly less fibrosis, reduction in apoptotic cardiomyocytes, and improved mitochondrial morphology compared to control hearts (Wang, et al. 2011b). Furthermore, mice with a targeted cardiac over-expression of CSE was protected against cardiac hypertrophy and left ventricular dysfunction (Calvert, et al. 2010).

c) H_2S and angiogenesis

Interestingly, although H_2S can inhibit VSMC proliferation, H_2S induces the proliferation of endothelial cells. NaHS (10 – 20 µM) was shown to increase migration, proliferation and induce tube-like structures in the RF/6A endothelial cell line and this effect was through activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) (Cai, et al. 2007). Moreover, the injection of NaHS (10 µmol/kg i.p.) over 7 days significantly increased neovascularisation *in vivo* (Cai, et al. 2007).

In another study, NaHS (300 µM) induced the expression of hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) mRNA in rat aortic VSMCs exposed to hypoxic conditions (Liu, et al. 2011). Moreover, the NaHS conditioned medium from the VSMC, induced the proliferation and migration of rat brain capillary endothelial cells; suggesting HIF-1α/VEGF is involved in H_2S-mediated angiogenesis under hypoxic conditions (Liu, et al. 2011).

H_2S (60 μM) was demonstrated to promote human umbilical vein endothelial cell (HUVEC) proliferation, migration and induced the formation of capillary tube-like structures; a mechanism that was dependent on K_{ATP} channel activation and p38 MAPK phosphorylation (Papapetropoulos, et al. 2009). Indeed, this promising effect of H_2S on angiogenesis requires further exploration.

d) H_2S and thrombosis/platelet function

NaHS has been shown to inhibit the aggregation of human platelets in response to a wide range of platelet aggregating agonists *in vitro* (Zagli, et al. 2007). The anti-aggregatory effect of H_2S was independent of potassium channel opening, cAMP, cGMP and $^{\bullet}NO$ (Zagli, et al. 2007). However, only high concentrations (IC_{50} of 0.70-5.62 mM, dependent on the platelet aggregating stimulus) of NaHS completely inhibited platelet aggregation (Zagli, et al. 2007), which may suggest endogenous H_2S is unlikely to unlikely to inhibit platelet aggregation.

Garlic may be a source of H_2S *in vivo* (Benavides, et al. 2007). In this study, human red blood cells (RBC) were capable of converting garlic polysulfides into H_2S; and this was dependent on a non-enzymatic pathway involving thiols compounds such as GSH, cysteine, homocysteine, *N*-acetyl-cysteine and reduced thiols on the RBC membrane (Benavides, et al. 2007). Moreover, H_2S derived from garlic is capable of relaxing PE-pre-contracted rat aortic rings in a dose dependent manner (Benavides, et al. 2007). In a double-blind, randomised, paired placebo-controlled trail, 14 healthy volunteers who took capsules of garlic oil (containing 9.9 g of garlic) had their blood taken 4 hours later (Wojcikowski, et al. 2007). The garlic oil did not reduce collagen- or ADP-induced platelet aggregation *in vitro* (Wojcikowski, et al. 2007). However, there

was a small but significant reduction (12%) in adrenaline-induced platelet aggregation from blood samples taken from volunteers taking garlic oil (Wojcikowski, et al. 2007). They concluded that an acute dose of garlic oil may not be beneficial in reducing platelet aggregation, however, it may be beneficial on chronic treatment (Wojcikowski, et al. 2007).

e) H_2S and the myocardium

H_2S has negative inotropic effects in the heart both *in vitro* and *in vivo* (Geng, et al. 2004). The perfusion of NaHS (3 mM) into the Langendorff heart perfusion model significantly inhibited left ventricular pressure development and reduced heart rate (Geng, et al. 2004). Moreover, injecting NaHS (2.8 µmol/kg i.v.) into rats induced a transient (60 sec) reduction in left ventricular pressure (Geng, et al. 2004). NaHS (100 µM) was shown to inhibit L-type calcium channel (I_{caL}) opening and reduce the action potential duration in rat ventricular cardiomyocytes (Sun, et al. 2008), and may be a reason for the negative inotropic effects of H_2S.

H_2S also protects the heart against myocardial ischemia and reperfusion, possibly through: stimulating the PI3/AKT/PKC pathway (Hu, et al. 2008b, Calvert, et al. 2010, Hu, et al. 2011); inducing K_{ATP} channel activation and activating PKC/ERK1/2 pathways (Hu, et al. 2008b); inhibiting Na^+/H^+ exchanger activity (Hu, et al. 2011); inducing nuclear localisation of nuclear factor-E2-related factor (Nrf2) (Calvert, et al. 2010); and preservation of mitochondrial structure and function (Elrod, et al. 2007). In general it appears that H_2S is predominantly protective in models of ischemia-reperfusion, which is also evident in ischemia-reperfusion models of the liver (Jha, et al. 2008, Kang, et al. 2009), kidney (Bos, et al. 2009, Xu, et al. 2009, Simon, et al. 2010),

lung (Fu, et al. 2008), retina (Biermann, et al. 2011) and intestines (Liu, et al. 2009, Henderson, et al. 2010).

Moreover, H_2S appears to be cardioprotective in a number of models including: cobalt chloride ($CoCl_2$) induced hypoxia *in vitro* (Yang, et al. 2011a); adriamycin-induced cardiomyopathy (Su, et al. 2009); metabolic inhibition (glucose-free buffer) in *vitro* (Pan, et al. 2006); and coxsakievirus B3 induced myocarditis (Hua, et al. 2009).

1.9.6. H_2S and inflammation

a) Local inflammation

The four cardinal signs of inflammation were first described by Celsus (30–38 B.C, ancient Rome) as *rubor* (redness), *calor* (heat), *dolor* (pain) and *tumour* (swelling/oedema) (Punchard, et al. 2004). Galen (130–200 A.D.) subsequently added loss of function (*functio laesa*) as part of the list of staple signs of inflammation (Punchard, et al. 2004). Inflammation is now described as a complex process involving the influx of inflammatory cells (leukocytes) controlled by a balance of mediators such as cytokines, complement, eicosanoids (e.g. leukotrienes and prostaglandins) and others. Acute inflammation is necessary for the healing and removal of harmful substances. Chronic or non-resolving inflammation, however, is a problem and may result in discomfort and the loss of function of normal tissue and lead to conditions such as osteoarthritis, multiple sclerosis, atherosclerosis and inflammatory bowel diseases. Leukocytes are a key component of the inflammatory cascade. To reach the site of inflammation, leukocytes must migrate from the blood into the tissues. In the process of leukocyte trafficking, selectins (e.g. E-selectin) are involved in the capture of leukocytes to the endothelial surface (Ehrhardt, et al. 2004). However, due to the low

affinity for their ligands results in a 'capture and release' motion resulting in the 'slow rolling' (<5 μm/second) of leukocytes along the endothelial cell surface (Ley, et al. 2007). The process of leukocyte firm adhesion and leukocyte arrest is as a result of the up-regulation of: α_4 and β_2 integrins (e.g. very late antigen-4 and lymphocyte function-associated antigen-1 respectively) on leukocytes and their respective ligands; adhesion molecules (ICAM-1 and VCAM-1) on endothelial cells (Ley, et al. 2007). Upon the firm adhesion to the endothelial cell wall, with the aid of other adhesion molecules such as platelet–endothelial-cell adhesion molecule (PECAM), vascular endothelial cadherin and junctional adhesion molecules (JAM), results in the migration of leukocytes through the endothelial barrier into the tissues (Muller 2003).

b) H_2S in models of inflammation

The role of H_2S in inflammation is unclear and extensive reviews on this topic of H_2S have been published (Li, et al. 2006, Wallace 2007, Olson 2011, Whiteman and Winyard 2011). A part of this thesis aims to examine the effects of H_2S in inflammation. Therefore, this section of the thesis will cover aspects that have not been covered in the discussion or introduction of Chapter 5.

Similar to $^{\bullet}NO$, a number of pro- and anti-inflammatory functions exist for H_2S. In a model of acute pancreatitis induced by caerulein, pre- and post-treatment with PAG (100 mg/kg i.p.) significantly reduced lung and pancreatic substance P concentrations and neurokinin-1 receptor mRNA, suggesting a pro-inflammatory effect of endogenous H_2S (Bhatia, et al. 2008a). Similarly, PAG (50 mg/kg i.p.) pre-treatment has been demonstrated to reduce rat hindpaw oedema and reduce myeloperoxidase (MPO, indicator of neutrophil infiltration) activity induced by carrageenan (Bhatia, et

al. 2005). Additionally, in a model of severe burn injury, both pre- or post- treatment with PAG (50 mg/kg i.p.) significantly reduced MPO activity in the lung and liver; whereas NaHS (10 mg/kg i.p.) injected at the time of burn injury, significantly increased MPO activity in the lung (Zhang, et al. 2010). NaHS (3 μmol/kg i.p.) pre-treatment has also been demonstrated to enhance neutrophil migration to the peritoneal cavity induced by LPS, in a K_{ATP} channel dependent mechanism (Dal-Secco, et al. 2008).

In contrast, pre-treatment with Na_2S or NaHS (100 μmol/kg, intragastric) inhibited leukocyte adherence in mesenteric venules induced by aspirin or fMLP (N-formyl-methionine-leucine-phenylalanine) through a K_{ATP} dependent mechanism (Zanardo, et al. 2006). Similarly, NaHS (100 μmol/kg i.p.) inhibited leukocyte infiltration in a rat model of carrageenan-induced inflammation of the air pouch (Zanardo, et al. 2006). A H_2S-releasing derivative of diclofenac (ACS15), but not diclofenac itself, significantly inhibited lung MPO activity on post- cerulein injection (Bhatia, et al. 2008b). Moreover, in a model of smoke and burn injury, subcutaneous injection of Na_2S (2 mg/kg) post-injury significantly reduced pro-inflammatory cytokine IL-1β and increased anti-inflammatory cytokine IL-10 in lung tissue (Esechie, et al. 2008).

Although, H_2S appears to have both pro- and anti-inflammatory effects in a number of models, H_2S appears to be predominantly anti-inflammatory in models of gastric injury (Fiorucci, et al. 2005a, Distrutti, et al. 2006a, Fiorucci, et al. 2007, Wallace, et al. 2007b, Wallace, et al. 2009). Whether there are organ responsive differences to H_2S is yet unclear.

c) Systemic inflammation

Sepsis is a systemic inflammatory disorder due to infection (e.g. bacteria, viruses or fungi) of the bloodstream (Doi, et al. 2009). Sepsis is a common disease state, with an estimated mortality rate of 28% in sever septic cases in the United States (U.S.) (Angus, et al. 2001). Sepsis has an average cost of $22,000 per case costing the U.S. healthcare system an estimated $16 billion each year (Angus, et al. 2001). Sepsis can be defined into two distinct haemodynamic phases. The first phase being the hyperdynamic phase (also termed warm shock), characterised a low systemic vascular resistance compensated by an increased cardiac output (Buras, et al. 2005, Doi, et al. 2009). During this phase, the innate immune system is activated through the binding of pathogen-associated molecular patterns to toll-like receptors (TLR) e.g. LPS to TLR-4 (Lu, et al. 2008). TLR activation results in the activation of downstream signaling pathways that eventually lead to the activation of transcription factors such as NF-κB (nuclear factor-κB), AP-1 (activator protein-1) and IRF-5 (interferon regulatory factor 5), and the subsequent production of pro-inflammatory cytokines and the activation of both immune and non-immune cell types (Lu, et al. 2008). The second later hypodynamic phase (cold shock) is characterised by a low systemic vascular resistance but with a reduced cardiac output (Buras, et al. 2005, Doi, et al. 2009). Shock is defined as a disturbance between oxygen delivery and oxygen consumption to tissues (Spronk, et al. 2004). A reduced cardiac output in the second haemodynamic phase of sepsis, leads to a reduction in oxygen delivery to tissues leading to septic shock and multiple organ failure (Spronk, et al. 2004). Septic shock is clinically defined as a mean systemic blood pressure of <60 mmHg after attempted fluid resuscitation (Doi, et al. 2009). As a physiological reflex to reduce these consequences and maintain systemic blood

pressure and oxygen delivery to vital organs such as the brain and heart, low priority areas such as the microvascular beds in the skin are shunted (Spronk, et al. 2004). If the microcirculation is not perfused in a timely manner, this can result tissue damage and eventually multiple organ failure (Spronk, et al. 2004). Multiple organ dysfunction is defined when two or more organs systems are impaired and homeostasis cannot be maintained without therapeutic intervention (Robertson and Coopersmith 2006). The consequences of reduced cardiac output and impaired microcirculatory perfusion are worsened by the activation of the clotting cascade, increased aggregability of leukocytes, endothelial adherence and increased blood viscosity (as a result of reduced deformability of red blood cells) leading to further oxygen deprivation to tissues (Astiz, et al. 1995, Spronk, et al. 2004).

d) H_2S in animal models of sepsis

Plasma $^{\bullet}NO$ is elevated in models of endotoxin shock and is thought to be one cause of the hypotension described in sepsis (Thiemermann and Vane 1990). Similarly, plasma H_2S is elevated in a rat model of endotoxin shock (Li, et al. 2005). Like iNOS, CSE expression is induced by LPS (Li, et al. 2005, Zhu, et al. 2010) and consequently CSE may be seen as pro-inflammatory. H_2S has been demonstrated to reduce blood pressure *in vitro* and *in vivo* (Zhao, et al. 2001), and it can be hypothesised that a rise in H_2S contributes to the hypotension induced by endotoxin shock. However, unlike inhibiting $^{\bullet}NO$ synthesis (Thiemermann and Vane 1990), inhibiting H_2S synthesis using PAG (up to 50 mg/kg) did not affect the MAP of septic rats (Collin, et al. 2005). However, in the same study PAG was shown to reduce serum aspartate aminotransferase and alanine transaminase, lipase and creatine kinase levels,

indicating a reduction in liver, pancreatic and neuromuscular injury respectively (Collin, et al. 2005). This suggests that inhibiting endogenous H_2S synthesis is beneficial in sepsis and these beneficial effects are independent of hemodynamic effects. Similarly in another study PAG (50 mg/kg i.p.) pre- and post- cecal ligation and puncture (CLP)-induced sepsis, significantly reduced MPO activity in the lung and liver; This is in contrast to NaHS (10 mg/kg i.p. injected at the time of CLP) which increased MPO activity in the lung and liver (Zhang, et al. 2006). Moreover, PAG treated mice had an increased survival rate compared to control (Zhang, et al. 2006). Another study utilising the same protocol as the latter demonstrated that PAG reduces and NaHS increases lung injury in CLP-induced sepsis through regulating the generation of the inflammatory neuropeptide substance P (SP) (Zhang, et al. 2007a). In a model of endotoxic shock, PAG (50 mg/kg i.p.) reduced lung and liver MPO activity, whereas NaHS (14 µmol/kg i.p) had the opposite effect and increased plasma tumor necrosis factor (TNF)-α (Li, et al. 2005). Evidence from the latter studies suggest both endogenous and exogenous H_2S elicits pro-inflammatory effects in models of sepsis.

H_2S has also been demonstrated to have anti-inflammatory effects in sepsis (Spiller, et al. 2010). PAG (50 mg/kg i.p.) pre-treatment increased TNF-α and macrophage inflammatory protein (MIP)-2 in the peritoneal exudates of CLP-septic mice (Spiller, et al. 2010). In addition, PAG was demonstrated to increase rolling and adhesion of leukocytes to the mesenteric venules of these septic mice; whereas NaHS (100 µmol/kg, subcutaneous) pre-treatment significantly reduced leukocyte rolling. (Spiller, et al. 2010) Moreover, NaHS pre-treatment significantly improved the mortality of CLP-septic mice (Spiller, et al. 2010). In another study, novel slow releasing H_2S donor GYY4137 (50 mg/kg, i.p. post-treatment) was shown to reduce the LPS-

induced hypotension in anesthetised rats (Li, et al. 2008b). Moreover, GYY4137 reduced plasma nitrite/nitrate, lung MPO, alanine aminotransferase, pro-inflammatory cytokines (TNF-α and interleukin (IL)-6) (Li, et al. 2008b). These data suggest that endogenous and exogenous H_2S is anti-inflammatory in models of sepsis.

The role of H_2S in inflammation is complex and appears to be contradictory. As a result, a part of my thesis will attempt to examine the effects of H_2S donors in models of inflammation.

1.10. Aims of thesis

1. To elucidate the mechanisms of endothelial cell derived H_2S by i) examining the release of H_2S from endothelial cells in response to agents that elevate intracellular Ca^{2+} ii) examining the Ca^{2+}/CaM dependence of CSE and CBS enzymes from liver homogenates.

2. To investigate the potential cross talk between $^{\bullet}NO$ and H_2S using $eNOS^{-/-}$ and $iNOS^{-/-}$ knockout mice by i) assessing organ H_2S synthesising activity and ii) examining CSE or CBS protein expression in different tissues.

3. To determine the effects of a novel slow releasing H_2S donor GYY4137 on i) endothelial cell adhesion molecule expression *in vitro* ii) to explore the mechanisms behind its effects on adhesion molecule expression and iii) investigate the effects of GYY4137 in carrageenan-induced mouse hindpaw oedema.

4. To identify and characterise ZJ802, a novel organosulfur compound. With the use of biochemical techniques i) examine the ability of ZJ802 to release H_2S and ii) determine its antioxidant capacity compared to NaHS and GYY4137. Determine whether ZJ802 has anti-inflammatory properties by investigating the effects of this drug in an *in vivo* mouse model of endotoxic shock.

CHAPTER 2 – General Materials and Methods

2.1. HUVEC cell culture

Umbilical cords of healthy normaltensive mothers were collected at the birth centre in St. Thomas NHS Foundation Trust. Cords were stored at 4^{o}C in cord pot solution (2.5 mM HEPES buffer, 1x HBSS, 387.5 µM gentamicin sulphate (PBS), 18.75 mM bicarbonate) until collection. Umbilical cords greater than 3 days old were discarded. Informed written consent was provided with all umbilical cords employed in these experiments. The umbilical vein was cannulated at both ends, and sterilised Dulbecco's phosphate buffered saline (PBS, room temperature) was injected to remove blood and to ensure no leakage occurred along the length of the cord. The umbilical vein was injected once with air to remove excess PBS. 20 ml collagenase (crude Type IA from Clostridium histolyticum, 1 mg/ml in serum free M199) was flushed through the vein using two 10 ml syringes connected at both ends of the cord. Collagenase is used to cleave the bond between glycine and the neutral amino acid (X) in the sequence Pro-X-Gly-Pro, to induce endothelial cell dissociation from the connective tissue of the umbilical vein. The cord containing the collagenase was wrapped in foil and placed in a 37^{o}C incubator for 10 min. After the incubation, the cord was subsequently massaged gently to aid removal of human umbilical vein endothelial cells (HUVEC). The collagenase subsequently spun down at 1200 rpm for 10 min and the pellet resuspended in full serum M199 (5 ml M199-20%: containing 10% v/v fetal calf serum (FCS), 10% v/v newborn calf serum (NCS), endothelial cell growth supplement (ECGS 5 ng/ml, heparin 0.175 mg/ml), L-glutamate 2 mM and penicillin 100 U/ml, streptomycin 0.1 mg/ml). The cell suspension was placed in a

gelatin (10 mg/ml) coated T25 flask and left overnight in the cell culture incubator ($37^{o}C$, 5% CO_2 and 95% air, humidified with $CuSO_4$). This culture was denoted as passage zero (P_o). The following day, the cells were rinsed twice with PBS to remove debris and fresh M199-20% was supplied.

In optimal cell culture conditions, cells will proliferate until cells come in contact with one another until no room is left to grow, this is termed contact inhibition. If the cells are over confluent (that is too packed) their growth will cease and proliferation will cease. To ensure enough cells are obtained for experimentation, cells will need to be split before proliferation ceases. Therefore upon 90-95% confluence, cells should be split into cell culture flasks with a greater area to grow or into more than one cell culture flask of a similar size.

Trypsin is a member of the serine protease family that is inactivated by Ca^{2+} or Mg^{2+}, and is used to re-suspend cells that adhere to cell culture flasks. To prevent cells from becoming over confluent, cultures were rinsed twice in Ca^{2+}/Mg^{2+} free PBS to remove excess serum (a source of Ca^{2+}) and 1 ml trypsin/EDTA (PAA, in Ca^{2+}/Mg^{2+} free PBS) was added into each flask. Under the light microscope, once the cells become rounded in shape and begin to lift off the flask, the flask was tapped gently and M199-20% was added to inactivate the trypsin. The cell suspension was subsequently divided into the appropriate number of flasks.

For HUVEC, once the T25 (P_0) was approximately 95% confluent, this culture was split directly into one T75 flask (termed passage 1, P_1). On 95% confluence of P_1, the T75 was split into three T75 flasks (P_2). HUVECs in P_2 were subsequently split into the final experimental conditions (P_3). To reduce cell differentiation into other cell types,

HUVECs greater than P_3 were not used. Endothelial cell cobblestone morphology was assessed under light microscopy before experiments were carried out.

During experimental conditions, a reduced serum medium was utilised (M199, 1% v/v FCS, penicillin 100 U/ml, streptomycin 0.1 mg/ml, 2 mM L-glutamine) to arrest and synchronise the cells in cell cycle phase G0 or G1.

2.2. BAEC Cell Culture

Primary bovine aortic endothelial cells (BAEC, Figure 2a) were kindly provided by Dr Richard Siow (King's College London, Department of Physiology). Cells were grown in full serum M199 (40% v/v FCS and L-glutamate (2 mM) containing 100 U/ml penicillin with 0.1 mg/ml streptomycin (PAA) in serum free M199 (supplemented with 18.75 µM bicarbonate). To maintain the cell culture, the cells were split using trypsin/EDTA (PAA) in a split ratio of 1:3 on 1% w/v gelatin (Biorad) coated flasks and maintained in the cell culture incubator. BAEC up to passage 10 were used in all experiments.

Cells were cryopreserved in M199-20% supplemented with 10% v/v DMSO (Method 2.4). Cells were defrosted in a water bath (37^{o}C) for approximately 3 min until the contents were defrosted. The vial of cells were immediately placed in 20 ml fresh M199-20% and left in the incubator overnight. The next day the medium was replaced with 10 ml fresh M199-20%.

2.3. RAW 264.7 Cell Culture

RAW 264.7 were purchased from the American Type Culture Collection (ATCC; TIB-71). RAW 264.7 is an immortalised murine macrophage cell line. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v FCS, L-glutamate 2 mM, penicillin 100 U/ml and streptomycin 0.1 mg/ml (DMEM-10%) in the

cell culture incubator. During experimental conditions serum free medium was used (SF-DMEM: 10% v/v water, L-glutamate 2 mM, penicillin 100 U/ml and streptomycin 0.1 mg/ml).

Cells were cryopreserved in DMEM-10% supplemented with 10% v/v DMSO (Method 2.4). Cells were defrosted in a water bath (37^{o}C) for approximately 3 min until the contents were defrosted. The vial of cells were immediately placed in 20 ml fresh DMEM-10% and left in the incubator overnight. The next day the medium was replaced with 10 ml fresh DMEM-10%.

2.4. Cryopreservation

Adherent cell lines were washed twice in warm PBS and lifted from the plate using Trypsin/EDTA and resuspended with fresh medium. Cells were centrifuged at 1200 g for 7 min. Medium was aspirated and resuspended with 1 ml full serum medium supplemented with 10% v/v DMSO. Cells were transferred to a cryogenic vial and placed in a “Mr Frosty” container (Nalgene). The Mr Frosty container was stored at -80^{o}C for a minimum of 1 night. For longer term storage (> 2 months), frozen cells were transferred from -80^{o}C to liquid nitrogen.

2.5. Bradford Protein Assay

Protein concentration was determined using the Quick Start Bradford Protein Assay according to the manufactures instructions. The basis of the Bradford protein assay is the binding of Coomassie Brilliant Blue G-250 dye to aromatic and basic (particularly arginine) amino acid residues in proteins. The dye exists in three forms: cationic (acidic, red: 470 nm), neutral (green: 650 nm) and anionic form (blue: 595 nm). The

cationic form of the dye donates a free proton to proteins containing side chains that are ionisable. This causes the protein to expose its hydrophobic sites and positive amine groups which bind to the hydrophobic and anionic part of the dye. As a result, once the dye binds to proteins it stabilises in the anionic (blue) form.

In brief, Bradford dye 250 µl and sample 5 µl were added to a 96-well plate and incubated at room temperature for at least 10 min. The standard curve was made up using bovine γ-globulin provided in the assay kit. The absorbance was then determined in a microplate reader (Spectra Max190) at 595 nm and analyzed using SOFTmax PRO (Version 3.0) software.

2.6. Bicinchoninic acid (BCA) Protein Assay

The Bradford protein assay (method 2.4.) is not compatible with reducing reagents as a result the bicinchoninic acid (BCA) protein assay (Thermo Scientific Pierce) was carried out where reducing reagents e.g. cell lysis buffer was used. The theory behind the assay is based on the reduction of cupric ion (Cu^{2+}) to cuprous ion (Cu^{+}) following binding to protein in an alkali environment. Two molecules of BCA are thought to bind to one cuprous ion to form a purple coloured product.

The BCA protein assay was carried out by mixing 50:1 of reagent A (containing bicinchoninic acid): reagent B (containing cupric sulfate). In a 96 well plate, working reagent 200 µl was mixed with of sample/standard 5 µl and allowed to incubate at room temperature for 30 min in the dark before absorbance was measured on a microplate reader at 562 nm. A standard curve was carried out using bovine γ-globulin from the Bradford protein standards.

2.7. Western Blotting and SDS-PAGE

2.7.1. Theory behind SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) is the process of separating proteins through a polyacrylamide matrix gel using an electric current. Proteins are sorted according to their size and/or charge, based on the principle that larger proteins migrate slower than smaller proteins. The pore size of the gel matrix that the proteins migrate through is determined by the percentage of acrylamide used. A higher percentage of acrylamide results in smaller pores that will impede the movement of larger proteins compared to smaller molecular weight proteins. Denatured proteins that are resolved in reducing conditions, that is in the presence of sodium dodecyl sulfate (SDS), is described as SDS-PAGE. Heating proteins in the presence of sample buffer containing SDS (a highly anionic detergent), results in the coating of negative charges in the unfolded proteins, as a result SDS-PAGE resolves proteins primarily according to their mass. This is in contrast to non-denaturing (native) PAGE that separates proteins with regard to their mass, shape of native structure as well as net charge. To ensure that the proteins running on SDS-PAGE have an overall net negative charge, the gels are immersed in electrophoretic running buffers that have an alkaline pH. Zwitterions are molecules that have both a negative and positive charge on the same molecule. Amino-acids in proteins are zwitterions as they have basic amine (NH_3^+) and an acidic carboxylic acid (COO^-) group. Immersing proteins in an alkaline pH neutralises the basic amine group ($NH_3^+ \rightarrow NH_2$) leaving the amino-acids with an overall negative charge from the carboxylic acid group, ensuring that the charge of a protein is not a variable in proteins running on SDS-PAGE. Reducing agents, such as β-mercaptoethanol are also added to the sample buffer to cleave disulfide bonds in

proteins, to ensure that no tertiary or quaternary structure remain in proteins running in SDS-PAGE. Diffusion during electrophoresis can blur the protein bands and it is therefore common to apply a stacking gel on top of the resolving gel (termed a discontinuous buffer system). The stacking gel, concentrates (or stacks) the proteins together before it is resolved (or destacked) in the resolving gel and provides increased resolution of the protein bands (Davis 1964, Ornstein 1964). The theory behind the stacking the proteins is the Cl^- in the gel buffer has the highest attraction to the anode and therefore migrates the fastest bringing it to the front of the moving boundary of proteins heading towards the anode (Davis 1964, Ornstein 1964). The glycine provided by the running buffer is only partially negative at the pH in the stacking gel and therefore has the slowest migration velocity towards the anode (Davis 1964, Ornstein 1964). The mobility of sample proteins in the gel migrate intermediate between the leading ion Cl^- and the trailing ion glycine (Davis 1964, Ornstein 1964). As the glycine front moves closer to the Cl^- front it concentrates and stacks the proteins into narrow zone. The change in pH and pore size at the boundary between the resolving and stacking gel causes the trailing ion to speed up and migrate through and past the sample proteins to migrate with the Cl^- ions. As the proteins escape the glycine/Cl^- front in the resolving gel, the proteins unstack according to their mass (Davis 1964, Ornstein 1964).

2.7.2. Theory behind Western blotting

Blotting refers to the transfer of a substance, either protein, RNA or DNA, onto a membrane. Western blotting (also known as protein immunoblot) is a method where proteins are transferred to a membrane and antibodies are used to detect and quantify the specific protein of interest. The electrophoretic transfer of proteins from

polyacrylamide gels to a membrane was first described by (Towbin, et al. 1979) and is now used as a standard procedure used to quantify proteins since. This is normally carried out in running buffer used in SDS-PAGE supplemented with methanol, termed transfer buffer. The methanol improves the transfer of proteins from gel to membrane and causes SDS to dissociate from proteins. (Pettegrew, et al. 2009) The membrane is subsequently blocked by incubation with a blocking buffer such as albumin or non-fat milk, before incubation with primary antibody to reduce non-specific binding of the antibody and hence reducing background a improving the signal to noise ratio. Excess primary antibody is subsequently washed off the membrane in a wash buffer consisting of a physiological buffer (such as PBS) containing a light detergent such as Tween-20. The protein of interest is usually quantitated through incubating the membrane with a secondary antibody conjugated to horse radish peroxidase (HRP). Excess secondary antibody is washed off and the membrane is subsequently incubated with a substrate for HRP, most commonly a chemiluminescent substrate. The light emitted from the chemical reaction of substrate and HRP is subsequently quantitated using film or a gel documentation (gel doc) system.

2.7.3. SDS-PAGE and Western blotting Protocol

Cells and tissues were lysed in lysis buffer (EDTA 5 mM, NaCl 150 mM, Tris-HCl 50 mM, 1% v/v Triton-X100, 1% w/v SDS, phenylmethylsulphonyl fluoride 1 mM (isopropanol), protease cocktail inhibitor (Sigma), Appendix B). Stacking gel and the appropriate resolving gel and was prepared (Appendix A) for SDS-PAGE. Protein samples in lysis buffer was mixed 1:1 with laemmli sample buffer (Appendix A) and subsequently denatured at 95^{o}C for 5 min. Denatured protein samples (10 µg / 25 µl) and molecular weight ladder (5 µl, GE healthcare RPN800E) were loaded into each well of the stacking

gel unless otherwise stated. The gel was subsequently run in running buffer (Tris-base 25 mM, Glycine 192 mM, SDS 0.1% w/v, pH 8.3) using the Bio-Rad mini gel system, at 110 V until the dye front reached the end of the plate. Proteins from the gel were then transferred to a polyvinylidene difluoride membrane (PDVF, 0.45 µm pore size, GE-Healthcare) using the mini-trans blot Electrophoretic Transfer Cell in cold transfer buffer (20% v/v methanol, Tris-base 25 mM, Glycine 192 mM, SDS 0.1% w/v, pH 8.3) at 130 V for 1 h. The PDVF membrane was subsequently blocked in 5% w/v non-fat milk in PBST (0.1% v/v Tween-20 in PBS) for 1 h. The membrane was subsequently incubated with primary antibody overnight at 4^{o}C (Appendix C). The membrane was subsequently washed 3x 10 min in PBST on a shaker before incubation with secondary-HRP linked antibody for 1 h at room temperature (Appendix C). After 1 h, the membrane was washed 3x 10 min in PBST and enhanced chemiluminescence (ECL) working reagent (Thermo Scientific, # 34080) was incubated with the membrane for 1 min before being developed in the gel documentation system (Syngene). The blots were subsequently quantified using ImageJ software.

2.8. Endpoint PCR for HUVEC

Genomic DNA is composed of nucleic acid sequences called introns and exons. Introns also known as 'intervening sequences', are not present in the final gene product. When RNA is transcribed from DNA, the RNA is processed, a term called splicing, where introns are removed and exons are joined to form messenger RNA (mRNA). The mRNA is used directly as a template by ribosomes to form proteins, and is an indicator of protein synthesis although not all mRNA is translated to protein. In summary, total RNA was extracted from HUVEC. Full length complementary DNA (cDNA) was reverse

transcribed from the total RNA extracted. The cDNA was subsequently amplified at the gene region of interest, CSE, as an indicator of CSE mRNA levels.

2.8.1. Total RNA extraction from HUVEC:

HUVEC were plated in 6 well plates (2.5 x10^5 cells/well) and treated with or without LPS (100 ng/ml, M199-1%) for 24 h. Medium was aspirated and Trizol (250 µl, Invitrogen) was immediately added to each well to lyse cells and stabilise RNA. Cells were homogenised on the plate using the back of a sterile, RNAse and DNAse free pipette tip and transferred to a sterile tube and frozen at -80^{o}C until use. To extract total RNA, the frozen Trizol samples were defrosted on ice and the isolation of RNA was carried out according to manufacturer's instructions (Invitrogen). In brief, chloroform (50 µl) was added to the Trizol sample, vortexed for 15 sec and incubated at room temperature for 5 min. The sample was centrifuged at 12,000 g for 15 min 4^{o}C. The upper aqueous phase containing the RNA was then aliquoted into DNA/RNAse free tubes. Isopropanol (125 µl) was added to the aqueous phase and incubated at room temperature for 10 min to precipitate the RNA. This was then centrifuged at 12,000 g for 10 min 4^{o}C. The RNA pellet was subsequently washed and resuspended in ethanol (250 µl, 75% v/v). This was subsequently centrifuged at 7500 g, 5 min, 4^{o}C. The ethanol wash was removed and the RNA pellet allowed to dry at room temperature for 10 min and subsequently resuspended in diethylpyrocarbonate (DEPC)-treated water (20 µl).

2.8.2. First-strand cDNA Synthesis:

Transcription is a physiological process in mammalian cells where RNA is synthesised from DNA, and is carried out by DNA polymerase. Reverse transcriptase is an enzyme, originally found in retroviruses, that is used to transcribe DNA from an RNA template and is termed reverse transcription or first-strand cDNA synthesis. DNA derived from RNA is termed complementary DNA (cDNA) and, in contrast to original full length genomic DNA, does not contain introns. To synthesise full length cDNA, RNA is reverse transcribed using oligo(dT) primers. Oligo(dT) primers work by annealing to the universal poly(A) sequences at the 3 prime (3') end of mRNA. (Nam, et al. 2002) Reverse transcriptase binds to the primer-mRNA template and begins the first stand of cDNA synthesis. The resulting cDNA is used as a template for PCR amplification (see later).

cDNA was synthesised by reverse transcription from the extracted total RNA using the First-Strand cDNA Synthesis kit (GE Healthcare). Commercial human liver RNA (AMS Biotechnology UK ltd: M1234149) was used as a positive control for CSE. Instructions were carried out as in manufacturer's instructions (GE Healthcare). In brief, total RNA (1 µg in 25 µl DEPC water) was denatured at 65^{o}C for 10 min to remove RNA secondary structure and chilled on ice for at least 2 min. Subsequently, the denatured RNA was added to commercial micro-centrifuge tubes with the first-strand reaction beads (containing Moloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase and nucleotides). Oligo(Dt) primers (1.65 µl, Fermentas) was added to the reaction mix to synthesise non-specific cDNA and a final volume of 33 µl was made up using DEPC water. This reaction was allowed to incubate at 37^{o}C for 1 h. After reaction, the cDNA was frozen -20^{o}C for PCR later. RNA and DNA quality and

concentration was quantified using the nanodrop spectrophotometer (Thermo Scientific) where absorbance ratio 260/280 nm for DNA ranged between 1.8 ± 0.2 and RNA between 2.0 ± 0.2.

2.8.3. Polymerase chain reaction (PCR):

PCR is a process where genes are amplified or replicated, enough for detection or identification purposes. The first stand cDNA synthesised from reverse transcription, is used in PCR as a template to amplify our gene of interest. Oligonucleotide primers were designed for genes of interest using published nucleotide sequences (NCBI nucleotide database). These primers anneal to the 3' or 5'end of the single strand of cDNA on the gene of interest, this occurs at an optimal temperature dependent on the primer nucleobases (cytosine, guanine, adenine and thymine). Optimal annealing temperature should be 3-5^{o}C below the primer melting temperature (T_m) determined broadly by:

$$T_m = 2(A + T) + 4(C + G)$$

Where: C = cytosine, G = guanine, A = adenine and T = thymine

Increasing the temperature from the annealing temperature to ~75^{o}C, the optimal activity of Taq DNA polymerase, (Lawyer, et al. 1993) causes the enzyme to bind the primers and synthesise another stand of DNA by adding complimentary nucleotides to the ends of each primer and using the single strand of DNA as a template. This generates a double stranded DNA in the region of interest. The duration of the 'extension' phase of the PCR reaction is dependent on the size of the gene of interest (typically 1 kilobase per minute). This cycle of heating and cooling, is repeated after heating the reaction mix to 95^{o}C, denaturing the double strands of DNA to form single

stranded DNA to again allow primer binding when the temperature is cooled to its annealing temperature. The end product consists of amplified double stranded DNA of your gene of interest.

Master mix (18 µl, Thermo scientific AB0575DCA, containing DNA Polymerase, nucleotides and $MgCl_2$ 1.5 mM), were added to DNAase free tubes containing cDNA (2 µg/µl in DNase/RNase free water), dimethyl sulfoxide (DMSO, 0.5 µl) and primers (0.5 µl in DNase/RNase free water, see Appendix D). DMSO was added to the reaction mix to reduce the formation of primer dimers. Settings that were used on the thermocycler (Techne, Cambridge, UK) for CSE, CBS and α-tubulin included: initial denaturation 94^{o}C for 5 min; followed by 40 cycles of denaturation at 94^{o}C for 30 sec, annealing at 57^{o}C for 40 sec and extension at 72^{o}C for 60 sec; and a final extension at 72^{o}C for 5 min and final hold at 10^{o}C. Heated lid was set at 105^{o}C. The predicted band for CSE, CBS and α-tubulin were calculated to be 157 bp, 150 bp and 200 bp respectively.

2.8.4. Agarose gel electrophoresis:

In order to visualise PCR product, agarose gels (1 % w/v, in 1x Tris-Borate EDTA (TBE) buffer, BioRad) containing ethidium bromide (1 µg/ml) were prepared. Gels were immersed in TBE buffer in a gel tank. PCR reactions (20 µl) were loaded onto the agarose gel alongside a 1Kb ladder (10 µl, Bioline) and run for 45 min at 70 V. The gel was subsequently visualised under UV light and the image captured in a gel documentation system (Syngene).

2.9. Methylene Blue method of H_2S detection

In this assay H_2S reacts with zinc acetate ($Zn(O_2CCH_3)_2$) to form zinc sulfide (ZnS). ZnS then reacts with the azo dye *N*,*N*-dimethyl-*p*-phenylenediamine (NNDPD), in the presence of acid and ferric chloride ($FeCl_3$) to form the coloured product methylene blue which is subsequently quantitated spectrophotometrically (Figure 2.9.1).

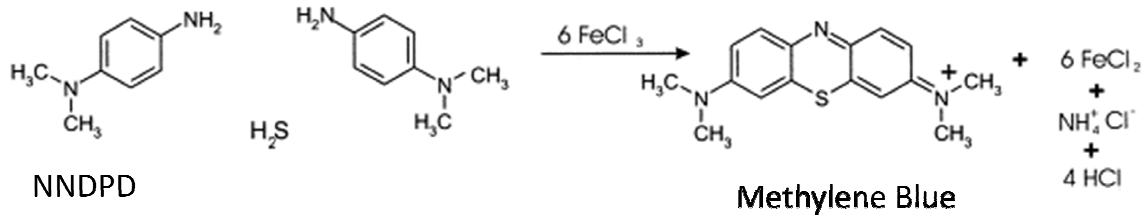

Figure 2.9.1. Methylene blue colourmetric method. Modified from (Lawrence, et al. 2000).

Culture medium (500 µl) was pipetted into an eppendorf tube containing zinc acetate (250 µl, 1% w/v in H_2O) and left for at least 5 min to complete the reaction with H_2S. Subsequently NNDPD dye (133 µl, 20 mM in 7.2 M HCl) followed by $FeCl_3$ (133 µl, 30 mM in 1.2 M HCl) was added to the zinc mixture and incubated at room temperature for 10 min. Thereafter, trichloroacetic acid (TCA, 250 µl 10% w/v in diH_2O) was added to stop the reaction and the tubes were centrifuged at 13,000 g at 4^{o}C for 4 min. This mixture 300 µl was pipetted into a microplate in duplicate and the absorbance read at 670 nm. A standard curve using sodium hydrogen sulfide (NaHS, 3.125 to 250 µM in medium) was used to determine the concentration of H_2S in the cell culture medium.

2.10. Sulfide Ion Selective Electrode of H_2S detection

The sliver/sulfide (Ag_2S) ion selective electrode is a solid state membrane electrode which can detect both the presence of free Ag^+ and S_2^- (Lazar research laboratories, CA). The electrode consists of an electrode body containing: a stable reference electrode, a filling solution (Ag_2S) and a sensing membrane that is immersed into the sample. The solid state sensing membranes are made from insoluble metallic salts containing the ion of interest (Pfafflin and Ziegler 2006). These membranes are ionic conductors and derive its selectivity through restricting the migration of non-specific ions therefore only allowing the movement of the specific ion within the lattice (Wang 2006). When the electrode is immersed in solution, sulfide molecules migrate through the membrane lattice until an equilibrium is reached between diffusion (high to low concentration) and electrostatic force (repulsion of molecules with similar charge) of the internal filling solution and the external sample solution (Wang 2006, Rundle 2008). The voltmeter subsequently calculates the potential difference derived from the reference electrode and the amount of S_2^- detected in the sample:

$$E = Eo + S*\log(A)$$

Where E = potential difference across the membrane, Eo = reference potential, S = electrode slope (i.e. the change in mV with a 10 fold change in concentration), A = effective concentration of sulfide, which is the product of the free sulfide ion concentration and the activity coefficient. The activity coefficient is dependent on the total ionic strength of the solution (aka the concentration and charge of all ions in solution) and is therefore variable between solutions to solution.

The sulfide ion selective electrode (ISE) was obtained from Lazar Research Laboratories, Los Angeles, California. The probe was utilised according to the manufactures instructions. Antioxidant buffer (final concentration: sodium salicylate 0.31 M, NaOH 2.13 M, ascorbic acid 0.37 M) was added to equal volume of sample to convert any form of free sulfide (HS^- and H_2S) into S_2^- which is detected by the ISE. Cell culture medium 500 µl was aliquoted and placed in 2-times concentrate antioxidant buffer 500 µl and measured directly using the sulfide ion selective electrode. The probe was calibrated using a standard curve using NaHS (0.1 – 500 µM) in antioxidant buffer.

2.11. Tissue H_2S synthesising assay

This assay is used to determine the P5'P and cysteine dependent enzymatic production of H_2S in tissues as an estimate of the relative activity and/or up-regulation of H_2S synthesising enzymes. To do this homogenised tissues were incubated with excess substrate cysteine and cofactor P5'P, at optimal enzyme conditions for 30 min at 37^oC, pH 7.4. H_2S concentration was used as the end point of the assay and detected through the methylene blue method of H_2S detection (Method 2.9).

Tissues from animals were snap frozen in liquid nitrogen and stored at -80^oC until use. Tissues were subsequently allowed to thaw completely on ice before being homogenised in cold $KHPO_4$ buffer (100 mM, pH 7.4) using a Polytron Homogeniser. The homogenate was then centrifuged at 13,000 g, 4^oC for 10 min. Supernatant from the homogenate 430 µl was incubated with $KHPO_4$ buffer control or treatment (30 µl), pyridoxal 5' phosphate (P5'P, 20 µl, 2 mM) and L-cysteine (20 µl, 10 mM). The tubes were covered with parafilm and the mixture was then allowed to incubate at 37^oC in

the waterbath for 30 min. All reactions were carried out in duplicate. As a background control, trichloroacetic acid (TCA, 250 µl of 10% w/v H_2O, Alfa Aesar) was added to the tissue supernatant to denature protein prior to the 30 min incubation period at 37^{o}C. After incubation, the samples were immersed in ice-cold water for 2-3 min to slow the rate of reaction and reduce the pressure built up in the tube. To trap H_2S produced, zinc acetate (250 µl of 1% w/v in H_2O) was injected and allowed to sit at room temperature for 5 min. To stop the reaction and precipitate protein TCA (250 µl of 10% w/v in H_2O) was subsequently added to the samples without TCA. Samples were centrifuged at 13,000 g, 4^{o}C for 4 min. *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride dye (NNDPD, 133 µl of 20 mM in 7.2 M HCl), followed by iron chloride ($FeCl_3$, 133 µl of 30 mM in 1.2 M HCl). The absorbance was then read at 670 nm in a 96-well microplate reader. H_2S concentration was calculated through the use of a NaHS standard curve (3.12-250 µM). Values were then standardised to protein content using the Bradford protein assay.

2.12. Statistical analysis

Data is expressed as mean ± SEM. A student's unpaired t-test was used to determine significance between two sets of data. When comparing two or more groups, a one-way analysis of variance (ANOVA) followed by a Dunnets post hoc test was carried out. A Dunnets test was carried out when comparing control to other variables. When comparing a time course, a two-way ANOVA was carried out followed by Bonferroni post hoc test. All statistics was carried out on GraphPad Prism (version 4). *P<0.05 was considered as significant.

CHAPTER 3: Cystathionine Gamma Lyase and The Endothelium

3. Introduction

Exogenous H_2S was first described to relax blood vessels in 2001 (Zhao, et al. 2001). In the same study, a bolus injection of H_2S was shown to induce a transient reduction in mean arterial blood pressure of anaesthetised mice which was inhibited by pre-treatment with glibenclamide, a K_{ATP} channel blocker (Zhao, et al. 2001). These authors also described the presence of CSE mRNA in the vascular smooth muscle cells, but not in the endothelium of rat aortic rings (Zhao, et al. 2001). Since then a vast amount of literature on the biological activity of H_2S within the cardiovascular system has been described. Blood pressure regulation by endogenous H_2S was conceivable to scientists working in the H_2S field, due to: the presence of H_2S synthesising enzyme activity in a number of vascular beds (Zhao, et al. 2001); and the presence of plasma H_2S in a number of vertebrates (Olson 2005, Yang, et al. 2008).

However, inhibitors of both CSE (Burnett, et al. 1980) and CBS (Rej 1977, Ochs and Harris 1980) are not very selective. Moreover, reported concentrations of H_2S in plasma were inconsistent between different laboratories, with reported levels from 2 µM (Olson 2009) to 280 µM (Han, et al. 2006) and the well documented toxicology of H_2S (Beauchamp, et al. 1984) caused speculation as to whether H_2S was really an endogenous signalling mediator or an artefact of the methods used to detect H_2S.

However, in 2008 there was solid evidence to demonstrate that CSE was involved in the physiological regulation of blood pressure (Yang, et al. 2008). $CSE^{-/-}$ KO mice were described to develop age dependent hypertension that was ~18 mm Hg greater than control at 12 weeks of age (Yang, et al. 2008). In addition, there was a

significant (*P<0.05) reduction in serum H_2S in the $CSE^{-/-}$ KO (~20 µM) compared to WT mice (~40 µM) (Yang, et al. 2008). Moreover, methacholine (MCh) induced relaxation of mesenteric arteries was markedly impaired in $CSE^{-/-}$ KO mice (Yang, et al. 2008). They also demonstrated that the hypertensive phenotype seen in the $CSE^{-/-}$ KO mice was not due to changes in: endothelial nitric oxide synthase (eNOS) protein levels; alterations in kidney architecture; accumulation of homocysteine; or increased levels of the superoxide anion in vascular tissue (Yang, et al. 2008). Interestingly, in contrast to their previous study (Zhao, et al. 2001), they showed the presence of CSE protein in the endothelial cell lining of wild type (WT) aortic rings which was absent in the $CSE^{-/-}$ KO mice (Yang, et al. 2008). Based on these results, the absence of CSE in the endothelium was hypothesised to be the cause of the endothelial cell dysfunction demonstrated with MCh in the mesenteric arteries of the $CSE^{-/-}$ KO mice (Yang, et al. 2008). To verify these results, they demonstrated the presence of CSE protein in cultured endothelial cells: bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) (Yang, et al. 2008). Furthermore, within 10 min of stimulating BAEC with calcium ionophore A23187 or MCh a measurable rise of H_2S in the culture medium was noted (Yang, et al. 2008). Moreover, using RNA interference to deplete CSE in the BAEC significantly reduced the H_2S produced *de novo* induced by MCh and A23187 by ~2-fold (Yang, et al. 2008). Exogenous H_2S has previously been demonstrated to relax a number of blood vessels (Zhao, et al. 2001). Together this evidence demonstrated that H_2S, like nitric oxide ($^{\bullet}NO$), is an endothelial cell derived relaxing factor (EDRF) and is released upon a physiological stimulus such as calcium (Ca^{2+}) (Yang, et al. 2008).

Hypothesis: H_2S is an endothelial cell derived relaxing factor that is induced upon a Ca^{2+} stimulus.

The primary aim of this project was to:

1. Examine the possibility of endothelium-derived H_2S release in response to a range of agonists that increase intracellular calcium (Ca^{2+}).
2. Examine the activity of H_2S synthesising enzymes using liver homogenates in the presence of calcium-calmodulin (CaM) antagonists.
3. Extend this study to assess the potential benefits of enhancing endothelium-derived H_2S in vascular biology.

3.1. Materials and Methods

3.1.1. Stimulating HUVEC and BAEC with A23187 and methacholine (MCh)

HUVEC (P_2-P_3) and BAEC (P_3-P_{10}) were plated in T25 flasks until 95% confluent. A23187 (1 – 10 µM), MCh (1 – 10 µM) (Yang, et al. 2008), bradykinin (10 µM) (Brodsky, et al. 2002), or vehicle (DMSO or acetic acid 0.1M, 1 µl per 1 ml) were added to the cells. H_2S was measured in the cell culture medium (M199-20%, M199-1% or serum-free (SF) M199, ± L-cysteine 500 µM) using the methylene blue colourimetric assay (Method 2.9) and the ISE (Method 2.10) after stimulation for 10 - 60 min. Using the methylene blue colourimetric method, zinc acetate (used to trap H_2S) was either injected directly into the cell culture medium (1.5 ml 1% w/v zinc acetate into 3 ml culture medium) of stimulated cells or the cell culture medium (500 µl) aliquoted into tubes containing zinc acetate (250 µl, 1% w/v H_2O). Several protocols were used to measure H_2S using the ISE: the probe was either put directly into the cell culture medium with the cells to measure H_2S production *de novo*; antioxidant buffer (Method 2.10) used to preserve sulfide, was directly injected into the cell culture medium (1:1, antioxidant buffer: cell culture medium) at the time point measured; at different time points cell culture medium from the stimulated cells were aliquoted into tubes containing antioxidant buffer.

3.1.2. H_2S enzyme activity assay in HUVEC and BAEC

Adherent cells from two T75 flasks (each 2 x 10^6 cells) of HUVEC P_3 or BAEC ($P_{4\text{-}10}$) were lifted from the plate with trypsin (Method 2.1) and the cell suspensions from both flasks were pooled together and centrifuged at 1000 g for 10 min. The cell pellet was

resuspended in 1 ml PBS and transferred to a 1 ml tube and centrifuged at 1000 g for 5 min. The PBS was removed and the cell pellet was frozen -20^{o}C.

The H_2S enzyme activity was carried out as in Method 2.11 with slight modifications. HUVEC pellets were defrosted on ice and manually homogensied in cold $KHPO_4$ buffer (260 µl, 100 mM pH 7.4). Mouse liver was used as a positive control and was homogensied again in the same way. The cell homogenates were centrifuged at 13,000 g for 4 min. 10 µl of the homogenised cell supernatant was reserved for the Bradford protein assay (Method 2.5). To the remaining cell supernatant, pyridoxial 5' phosphate (P5'P 10 µl 2mM in $KHPO_4$ 100 mM pH 7.4) and L-cysteine (10 µl 10 mM in $KHPO_4$ 100 mM pH 7.4) was added. The tubes were tightly covered with parafilm and the reaction was allowed to proceed at 37^{o}C in the waterbath for 1 h. After incubation, the samples were immersed in ice-cold water and zinc acetate was injected into each tube (125 µl, 1% w/v H_2O), followed by trichloroacetic acid (TCA, 125 µl, 10% w/v H_2O). Samples were centrifuged at 13,000 g for 4 min. NNDPD (66.5 µl 20 mM in HCl 7.2 M) followed by $FeCl_3$ (66.5 µl, 30 mM in HCl 1.2 M) was subsequently added to each of the tubes. After at least 15 min incubation at room temperature, the absorbance was read at 670 nm in a 96-well microplate reader.

3.1.3. Measurement of intracellular Ca^{2+} using Fura-2AM

Intracellular Ca^{2+} responses to A23187 (1 µM), MCh (10 µM) or bradykinin (BK, 10 µM) were measured in BAEC or HUVEC using Fura-2 acetoxymethyl ester (Fura-2AM). In brief, endothelial cells were plated on the centre of glass coverslips and grown in full serum M199 until confluence. On the day of the experiment, cells were loaded with pluronic acid (0.3 mg/ml in DMSO) and Fura-2AM (2.5 µM in DMEM containing 20%

FCS and buffered with HEPEs free acid) and incubated for 1 h in the dark at room temperature. Population experiments were performed in which the global intracellular Ca^{2+} response was measured from many individual cells. The coverslips were placed diagonally inside a cuvette containing warm (37^{o}C) balanced salt solution (NaCl 145 mM, KCl 5 mM, $MgSO_4$ 1 mM, HEPES 10 mM) to which A23187 (1 μM), MCh (10 μM) or BK (10 μM) was added once the background was stable. A spectrophotometer (Cairn Research Ltd, Faversham, UK) with a rotating wheel equipped with 340 nm and 380 nm filters were used to excite the Fura-2 and emission was subsequently detected at 510 nm. ATP (100 μM) was used as a positive control for cell responsiveness. Before terminating the experiment, digitonin (40 μM) was used to permeabilise the cells to allow influx of extracellular Ca^{2+} to determine the maximal Ca^{2+} fluorescence. Subsequently, manganese chloride ($MnCl_2$, 6 mM) was used to quench the Fura-2 fluorescence to determine background autofluorescence. Fura-2 displays an absorption shift when the excitation wavelength changes between 300-400 nm while observing the emission at 510 nm. This useful property of Fura-2 allows the ratio of the absorbance when excited by two excitation wavelengths (e.g. 340 nm and 380 nm) to be calculated. Taking the ratio at two excitation wavelengths instead of an absolute value at one wavelength has its advantages: it can eliminate uneven distribution of the sensor, uneven dye loading, photobleaching, dye leakage and variations in cell thickness (Takahashi, et al. 1999). The ratio is normally taken at excitation wavelength of 340 nm (where the emission is at its peak) and at 380 nm (where the emission is declining). A change in intracellular Ca^{2+} was represented as a ratio of the emission at 340:380 nm.

3.1.4. H_2S quenching studies

NaHS (1 mM, 500 µl) was incubated with copper sulfide ($CuSO_4$, 1 mM, 500 µl), magnesium chloride ($MgCl_2$, 1 mM, 500 µl), calcium chloride ($CaCl_2$, 1 mM, 500 µl), iron chloride ($FeCl_3$, 1 mM, 500 µl), haemoglobin (Hb, 1 mM, 500 µl), unheparinised lysed rat whole blood, bovine albumin (2.5 mg/ml, 500 µl) and fetal calf serum (FCS, 500 µl). H_2S was measured using either the methylene blue method of detection (Method 2.9) or the sulfide ISE (Method 2.10).

3.1.5. Stimulating HUVEC and RAW264.7 with LPS to induce CSE protein expression

Lipopolysaccharide (LPS) has been shown to stimulate CSE expression in RAW264.7 and in the tissues of LPS treated mice (Li, et al. 2005, Zhu, et al. 2010). HUVEC or RAW264.7 cells were plated on 6-well plates and treated with 100 ng/ml or 1 µg/ml LPS (Escherichia coli 055:B5) in M199-1% or SF-DMEM respectively for 24 h (Biffl, et al. 1996, Whiteman, et al. 2010c). Cells were subsequently rinsed twice in PBS and lysed with lysis buffer (50 µl) (Appendix B) and frozen at -20^{o}C for CSE protein measurement by western blot later. Using the methylene blue H_2S detection method (Method 2.9), H_2S in the cell culture medium was measured in LPS stimulated RAW264.7 after 24 h.

3.1.6. Western blot for CSE and CBS protein

Western blot was carried out as in Method 2.7. In brief, 10 µg protein/well (HUVEC, RAW264.7 or mouse liver) was resolved on a 7.5% gel and transferred to a PDVF membrane (wet transfer). The membrane was incubated with 5%w/v non-fat milk for 1 h prior to incubation with antibody for CSE, CBS or β-actin (Appendix C) overnight at 4^{o}C. The PDVF membranes were washed in PBS-tween for 30 min prior to incubation

with secondary-HRP linked antibody for 2 h (Appendix C). The membranes were further washed for 30 min and subsequently incubated with ECL working reagent for 1 min prior to visualisation on a gel documentation system (Syngene). The molecular weight band described for CSE, CBS and β-actin were 43 kDa, 75 kDa, and 42 kDa respectively.

3.1.7. Endpoint PCR for CSE and CBS messenger RNA

HUVEC were plated on 6-well plates and treated with or without LPS (100 ng/ml, Escherichia coli 055:B5) in M199-1% for 24 h. At the end of the incubation period, Trizol (250 µl, Invitrogen) was added to each well, the cells homogenised and samples frozen at -80^{o}C. Total RNA was extracted (Method 2.8.1) and first strand cDNA synthesis was carried out (Method 2.8.2). Endpoint PCR was conducted as described in Method 2.8.3. In brief, a master mix (18 µl, Thermo scientific AB0575DCA), was added to DNAase free tubes containing human liver or HUVEC cDNA (2 µg/µl), DMSO (0.5 µl) and primers (0.5 µl, for human CSE, CBS and α-tubulin, see Appendix D). cDNA was amplified according to the following settings: initial denaturation 94^{o}C for 5 min; followed by 40 cycles of denaturation at 94^{o}C for 30 sec, annealing at 57^{o}C for 40 sec and extension at 72^{o}C for 60 sec; and a final extension at 72^{o}C for 5 min and final hold at 10^{o}C. Heated lid was set at 105^{o}C.

In order to visualise DNA, agarose gels (1 % w/v, in 1x TBE buffer) containing ethidium bromide (1 µg/ml) were prepared. PCR reactions (20 µl) were loaded onto the agarose gel alongside a 1Kb ladder (10 µl, Bioline) and run for 45 min at 70 V. The gel was subsequently visualised under UV light and the image captured in a gel

documentation system (Syngene). The predicted band for CSE, CBS and α-tubulin were calculated to be 157 bp, 150 bp and 200 bp respectively.

3.1.8. Ca^{2+}/CaM dependence of H_2S enzymes in liver homogenates

H_2S enzyme activity assay in liver was carried out as in Method 2.11 with the following modifications. Liver homogenates (5% w/v) were homogenised in cold $KHPO_4$ buffer (100 mM, pH 7.4). The homogenate was then centrifuged (13,000 g, 4^{o}C for 10 min) and the supernatant (430 µl) was incubated with pyridoxal 5' phosphate (P5'P, 20 µl 2 mM), L-cysteine (20 µl of 10 mM), treatment (30 µl $KHPO_4$ buffer control, trifluoperizine 8.3 – 125 mM, $CaCl_2$ 8.3 mM or EGTA 100 mM). The tubes containing the reaction mix were covered with parafilm and allowed to incubate at 37^{o}C in the waterbath for 1 h. Zinc acetate (250 µl of 1% w/v in H_2O) was subsequently injected into the tubes and allowed to sit at room temperature for 5 min to convert sulfide produced by the liver homogenates into zinc sulfide. TCA (250 µl of 10% w/v in H_2O) was subsequently added to stop the reaction and precipitate protein. Samples were centrifuged (13,000 rpm, 4^{o}C for 4 min). Zinc sulfide was subsequently quantitated using the methylene blue colourimetric method where AZO dye NNDPD (133 µl of 20 mM in 7.2 M HCl) and $FeCl_3$ (133 µl of 30 mM in 1.2 M HCl) was added to each sample and incubated at room temperature for at least 10 min. The absorbance was read in a 96-well plate at 670 nm. Values were then standardised to protein content using the Bradford protein assay (Method 2.5). H_2S concentration was calculated through the use of a NaHS standard curve (3.12-250 µM)

3.2. Results

3.2.1. Comparing the ISE and methylene blue method of H_2S detection

Initially, the limit of detection of H_2S using the ISE and the methylene blue assays were characterised and compared. Varying concentrations of NaHS (0.1 – 500 μM) were made up in water and incubated with either zinc acetate (methlyene blue colourimetric method) or antioxidant buffer (ISE). The lower limit of H_2S detection in antioxidant buffer using the ISE was approximately 10 μM NaHS and the linear working range was between 10 – 50 μM NaHS (Figure 3.2.1 a and b). In contrast, the methylene blue method of H_2S detection was more sensitive and had a greater breadth of H_2S detection with a linear working range from ~3 – 100 μM NaHS (Figure 3.2.1 c and d).

3.2.2. Measuring H_2S synthesis *de novo* from HUVEC and BAEC

In contrast to previous reports (Yang, et al. 2008), stimulating BAEC or HUVEC with A23187 (1 μM), MCh (10 μM) or bradykinin (10 μM) for 10 min did not result in a measurable rise of H_2S in the cell culture medium using both the methylene blue (Figure 3.2.2 b and d) and the ISE (Figure 3.2.2 a and c) methods of H_2S detection. Moreover, increasing the time point at which H_2S was measured (up to 60 min) did not lead to detectable amounts of H_2S released from BAEC stimulated with A23187 (Figure 3.2.3). Interestingly, homogenising HUVEC or BAEC and incubating them with substrate cysteine and co-factor pyridoxal 5’ phosphate demonstrated low levels of H_2S synthesising activity in both cell lines (Table 3.2.1), suggesting the presence of H_2S synthesising enzymes in these endothelial cells.

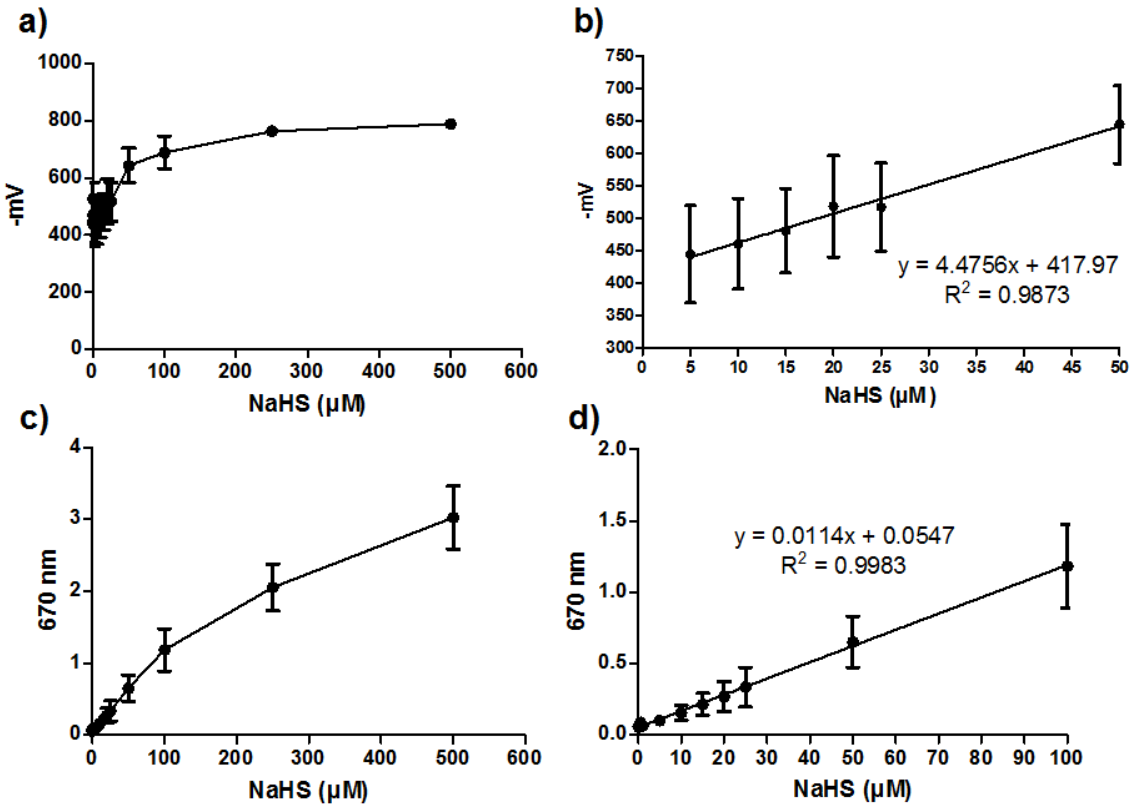

Figure 3.2.1. Standard curves for NaHS using the: sulfide ion selective electrode (ISE) a) 0.1 – 500 µM b) 5 – 50 µM; methylene blue colourimetric assay c) 0.1 – 500 µM d) 0.1 – 100 µM. Results show mean ± SEM, n = 4

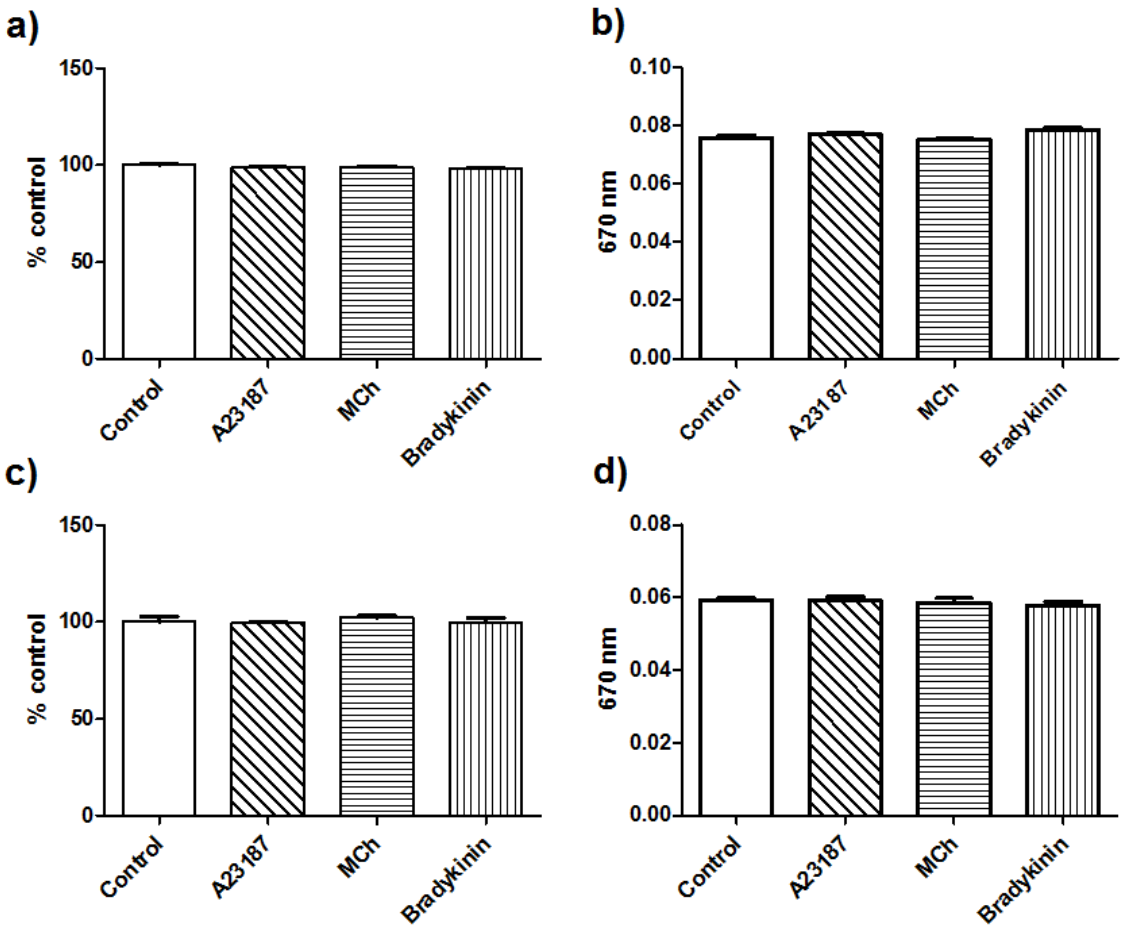

Figure 3.2.2. HUVEC or BAEC were stimulated with A23187 (1 µM), MCh (10 µM) or bradykin (10 µM) for 10 mins and H_2S in the medium (M199 containing 1% v/v fetal calf serum) was measured using the sulfide ISE a) and c) or via methylene blue colourimetric assay b) and d). Absolute H_2S concentrations for control were 0 µM H_2S for all assays. Results show mean ± SEM, n = 3.

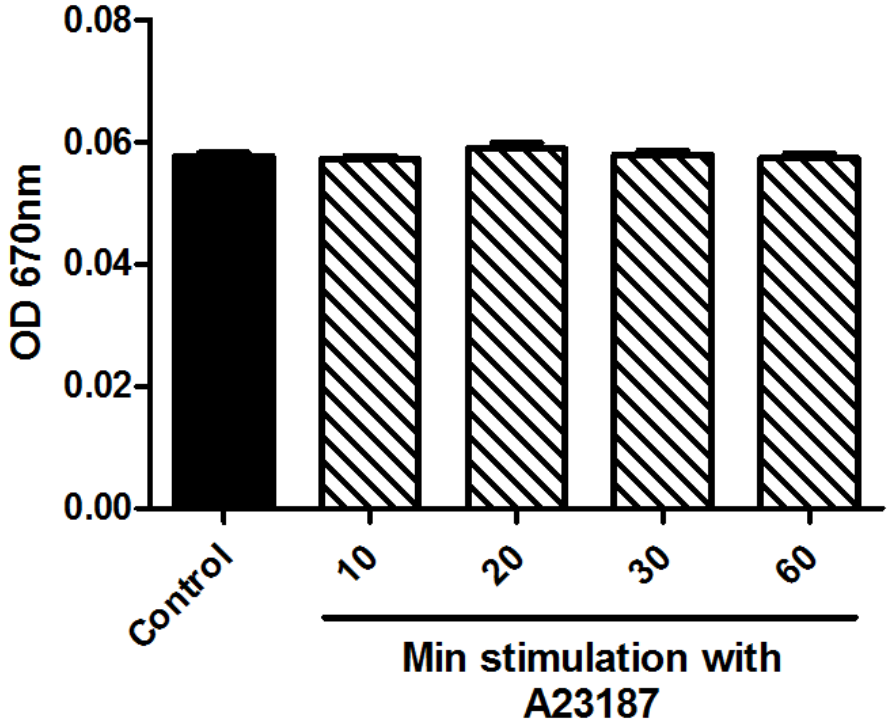

Figure 3.2.3. BAEC were stimulated with A23187 (1 µM) for 10 – 60 mins. At each time point H_2S was measured in the cell culture medium (M199 containing 1% v/v fetal calf serum) using the methylene blue colourimetric assay. Absolute H_2S concentrations for all time points were 0 µM H_2S. Results show optical density at 670 nm, mean ± SEM, n = 3.

Table 3.2.1. Typical values of H_2S synthesising activity in different tissues done on separate occasions. Tissues were homogenised and incubated with cysteine and pridoxal 5' phosphate for 1 h at 37^{o}C. H_2S produced was detected by the methylene blue colourimetric assay and used as an end point to estimate the H_2S enzyme activity. Results show mean ± SEM of H_2S nmol/mg protein.

Tissue	**H_2S nmol/mg protein**
HUVEC	0.506 ± 0.070, n = 7
BAEC	0.910 ± 0.236, n = 5
Rat liver	18.334 ± 0.515, n = 4

3.2.3. Calcium mobilisation in BAEC in response to A23187, MCh and BK

To ensure that Ca^{2+} was elevated in BAEC treated with A23187 (1 µM), MCh (10 µM) or BK (10 µM), BAEC were loaded with Fura-2AM, a tool used to detect a rise in intracellular calcium (Ca^{2+}). A23187 and BK induced an almost immediate measurable rise in florescence ratio, indicating a rise in intracellular Ca^{2+} (Figure 3.2.4 a and c).

Interestingly, after 10 min MCh did not cause a rise in intracellular Ca^{2+} in BAEC (Figure 3.2.4 b). However, in the same cells, ATP (100 µM) positive control could induce a rise in intracellular Ca^{2+} indicating that the cells were viable and responsive to agonist stimulation (Figure 3.2.4 b). These results suggested that BAEC were responsive to A23187 and BK, but not MCh.

3.2.4. H_2S quenching in the medium

To examine whether a component in the cell culture medium quenched the free unbound H_2S, standard curves using NaHS were made up in different culture medium (M199 or phenol-red free DMEM) containing various amounts of fetal calf serum (1% v/v or 20% v/v) (Figure 3.2.5). Both types of medium containing high concentrations of serum (20% v/v) significantly reduced the amount of H_2S detected using the methylene blue colourimetric method (Figure 3.2.5).

To investigate this further, components found in cell culture medium and/or the body tissue (Cu^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, haemaglobin, rat whole blood, albumin and FCS) were incubated with equimolar NaHS (500 µM). 500 µM NaHS was chosen as a large measurable amount of H_2S, to determine the extent of H_2S quenching. Both the methylene blue method (Figure 3.2.6) the ISE (Figure 3.2.7) indicated that H_2S was significantly quenched by copper (Cu^{2+}), iron (Fe^{3+}), haemogloblin (Hb), rat whole blood and fetal calf serum (FCS). Fe^{3+} is present in trace amounts in culture medium.

Therefore it is possible that low levels of H_2S produced by endothelial cells are quenched by Fe^{3+}. However, previous studies have successfully measured H_2S in cell culture medium (Yang, et al. 2008). In the current studies, 1%v/v FCS was utilised in the studies to measure H_2S release from endothelial cells in response to A23187, BK and MCh. It is possible the use of 1%v/v FCS quenched the H_2S release from the endothelial cells.

3.2.5. Measuring H_2S synthesis *de novo* from HUVEC and BAEC in the presence of L-cysteine

To guarantee the H_2S was not quenched by serum, HUVEC and BAEC were stimulated with A23187 (1 µM) or BK (10 µM) in serum-free medium. Utilising lower passage HUVEC (P_2) and BAEC (P_{3-5}) together with the serum-free conditions did not lead to a measurable amount of H_2S detected by the methylene blue assay (Figure 3.2.8 a and c). Furthermore, pre-treating these cells with L-cysteine (500 µM) (Furne, et al. 2008) for 1 h prior to stimulating HUVEC or BAEC with A23187 or BK for 10 min also did not lead to detectable amounts of H_2S in the culture medium (Figure 3.2.8 b and d).

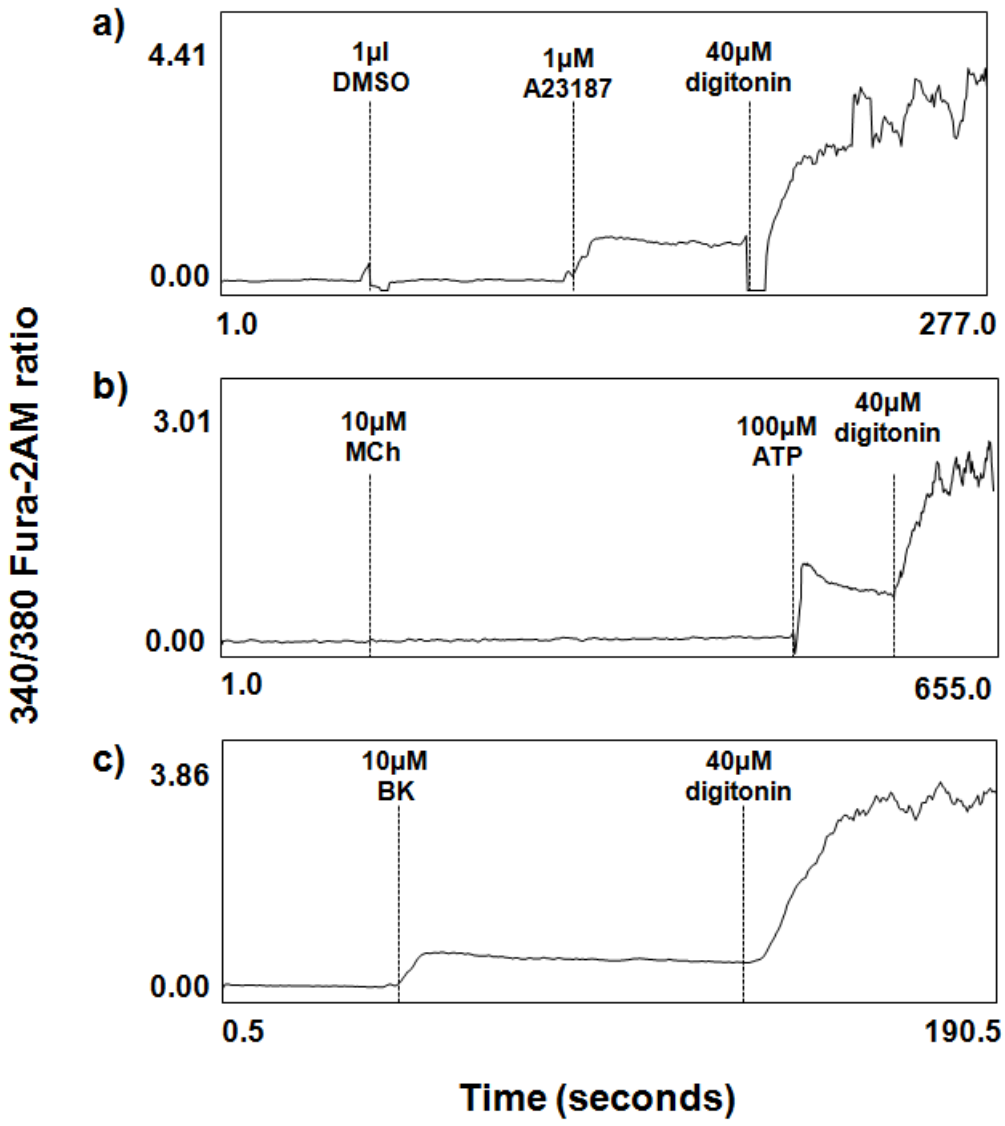

Figure 3.2.4. BAEC were loaded with Fura-2AM for intracellular Ca^{2+} detection and stimulated with DMSO (1 µl), A23187 (1 µM), methacholine (MCh, 10 µM), bradykin (BK, 10 µM) or ATP positive control (100 µM). Digitonin (40 µM) was used to determine the maximum intracellular Ca^{2+} load. Each trace is representative of 3 separate experiments.

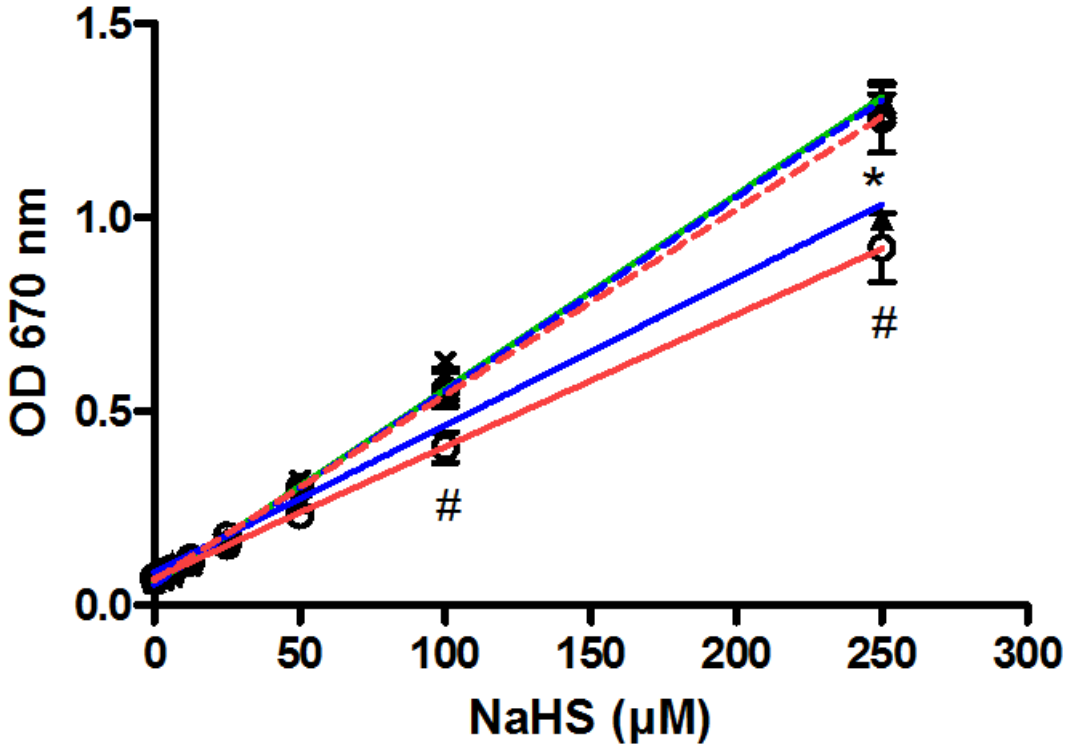

Figure 3.2.5. NaHS standard curves (3.125-250 µM) were carried out using water (H_2O, green solid line), Medium-199 with 1% v/v serum (M199-1%, red dashed line), M199 with 20% v/v serum (M199-20%, red solid line), Dulbecco's Modified Eagle Medium (DMEM without phenol red) with 1% v/v serum (DMEM-1%, blue dashed line) or 20% v/v serum (DMEM-20%, blue solid line). H_2S was measured using the methylene blue colourimetric assay. Results show optical density at 670 nm, mean ± SEM, n = 3. *P<0.05 H_2O c.f. DMEM-20%, #P<0.05 H_2O c.f. M199-20%

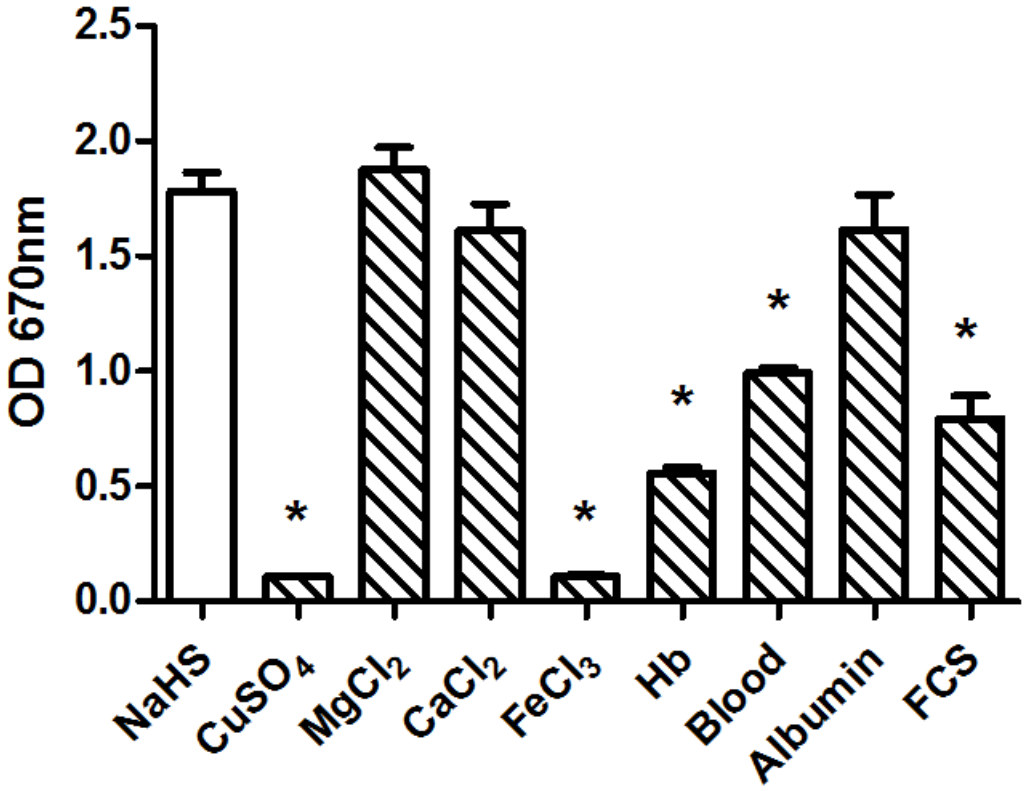

Figure 3.2.6. NaHS 500 µM was incubated with 500 µM copper sulfide ($CuSO_4$), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), iron chloride ($FeCl_3$), haemoglobin (Hb), unheparinised lysed rat whole blood, bovine albumin (2.5 mg/ml) and fetal calf serum (FCS). H_2S was measured using the methylene blue method of H_2S detection. Results show mean ± SEM, n = 6. *P<0.05 c.f. NaHS control.

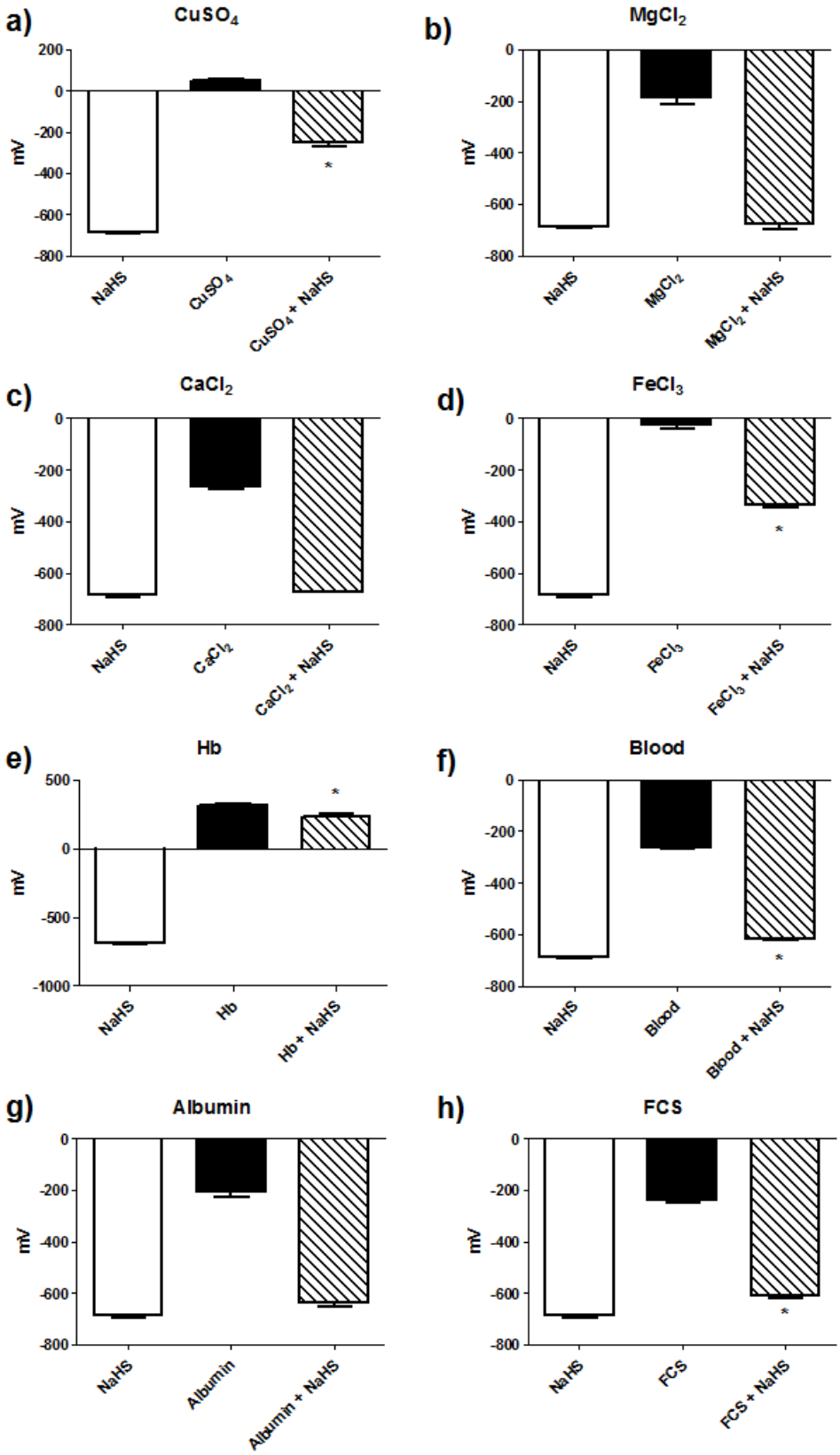

Figure 3.2.7. Sulfide ion selective electrode (ISE) measuring NaHS (500 µM, 500 µl), quenching substance (500 µM or 500 µl) or quenching substance + NaHS, in millivolts (mV). Quenching substance being a) copper sulfate ($CuSO_4$), b) magnesium chloride ($MgCl_2$), c) calcium chloride ($CaCl_2$), d) iron chloride ($FeCl_3$) e) haemoglobin (Hb) f) rat whole blood (blood, 500 µl) g) albumin (2.5 mg/ml, 500 µl) h) fetal calf serum (FCS, 500 µl). Results show mean ± SEM, n = 6. *P<0.05 c.f. NaHS control.

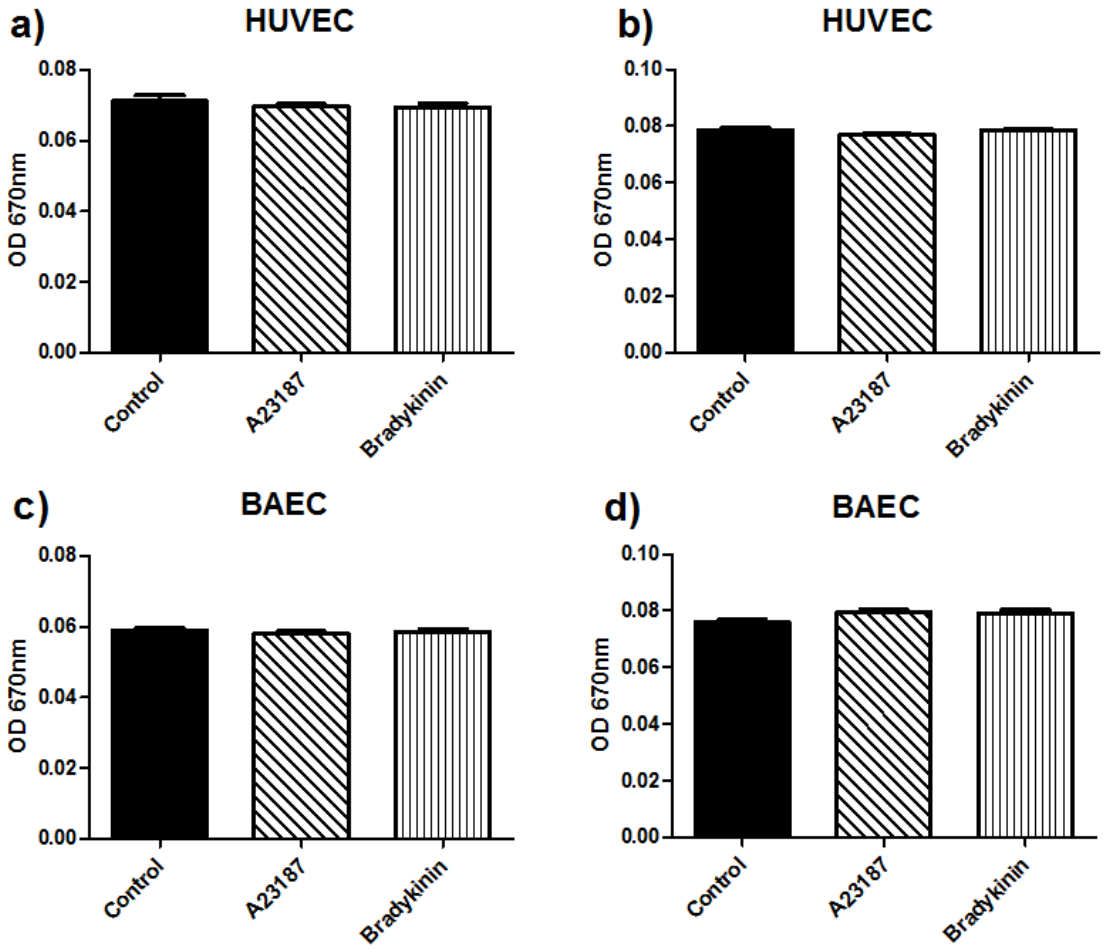

Figure 3.2.8. HUVEC (P_2) or BAEC ($P_{3\text{-}5}$) were stimulated with A23187 (1 µM) or bradykinin (10 µM) and H_2S was measured in cell culture medium after 10 min using the methylene blue colourimetric assay. a) HUVEC or c) BAEC were stimulated in serum free M199 b) HUVEC or d) BAEC were pre-treated with L-cysteine (500 µM, serum free M199) 1 h prior to stimulation. Absolute values for all data represent 0 µM H_2S. Results show optical density at 670 nm, mean ± SEM, n = 3.

3.2.6. CSE and CBS mRNA and protein expression in HUVEC

HUVEC were examined for CSE or CBS mRNA (end point PCR) and protein. HUVEC contained both CSE and CBS mRNA (Figure 3.2.9). However, neither CBS (Figure 3.2.10) nor CSE (Figure 3.2.11) protein was detectable in HUVEC. Furthermore, LPS stimulated HUVEC did not express CSE protein (Figure 3.2.11).

3.2.7. CSE protein expression in LPS stimulated RAW264.7

CSE protein expression was up regulated in RAW264.7 macrophages stimulated with LPS (1.0 μg/ml) (Figure 3.2.11). Although CSE was significantly up regulated in LPS stimulated RAW 264.7 cells, H_2S was not detected in the culture medium of these macrophages at 24 h using the methylene blue H_2S detection method (Figure 3.2.12).

3.2.8. H_2S-synthesising Activity in Liver Homogenates ± Calcium

The Liver has been reported to be a large source of the H_2S synthesising enzymes, CSE and CBS, with detectable amounts of H_2S produced in the presence of substrate (L-cysteine) and cofactors (pyrdoxial 5' phosphate, P5'P) under optimal enzyme conditions (Li, et al. 2005, Kabil, et al. 2011). Indeed, in the current study, measurable amounts of H_2S was detected from rat liver tissue on incubation with L-cysteine and P5'P (Figure 3.2.13). Smaller measureable amounts of H_2S were detected in the absence of P5'P, but not in the absence of L-cysteine (Figure 3.2.13 a) suggesting L-cysteine is the rate limiting factor in this reaction.

Incubating liver homogenates with CSE inhibitor with DL-propargylglycine (PAG, 60 μM, % inhibition 24.170 ± 1.100) or CBS inhibitor aminooxyacetic acid (AOAA, 60 μm, % inhibition 88.729 ± 0.458) resulted in a significant ($P<0.05$) reduction in H_2S

production (Figure 3.2.13 b) suggesting these enzymes are involved in the generation of H_2S in these liver homogenates.

To investigate whether these liver H_2S synthesising enzymes were calcium-calmodulin (Ca^{2+}/CaM) dependent, H_2S synthesising activity (as before) was carried out in the presence of either: Ca^{2+}/CaM competitive antagonist trifluoperizine (TFP, 500 – 7500 µM); added Ca^{2+} ($CaCl_2$); or Ca^{2+} chelator EGTA. Tissue H_2S synthesising activity was significantly (*P<0.05) inhibited at exceptionally high concentrations of TFP 5 mM (Figure 3.2.14 a, % inhibition 18.382 ± 4.680), which may suggest either a Ca^{2+}/CaM dependent component in H_2S enzyme activity or a non-selective effect of the drug.

The addition of Ca^{2+} (500 µM) alone to liver homogenates did not significantly increase H_2S enzyme activity (Figure 3.2.14 b). This may suggest H_2S enzymes are not Ca^{2+}/CaM dependent or Ca^{2+} is already in excess and therefore not a rate limiting factor in H_2S biosynthesis in the liver. However, adding Ca^{2+} chelator EGTA (6 mM) did not significantly reduce H_2S enzyme activity in these liver homogenates (Figure 3.2.14 b).

To ensure PAG, AOAA or TFP did not interfere with the colour change associated with the methylene blue colourimetric method, H_2S donor NaHS was incubated with PAG, AOAA or TFP and the assay was carried out as usual. None of the inhibitors PAG, AOAA or TFP interfered with the colour change associated with the methylene blue assay (Figure 3.2.15). This suggests that these inhibitors do not interfere with the colour development in the assay and are instead directly inhibiting enzyme activity in the liver homogenates.

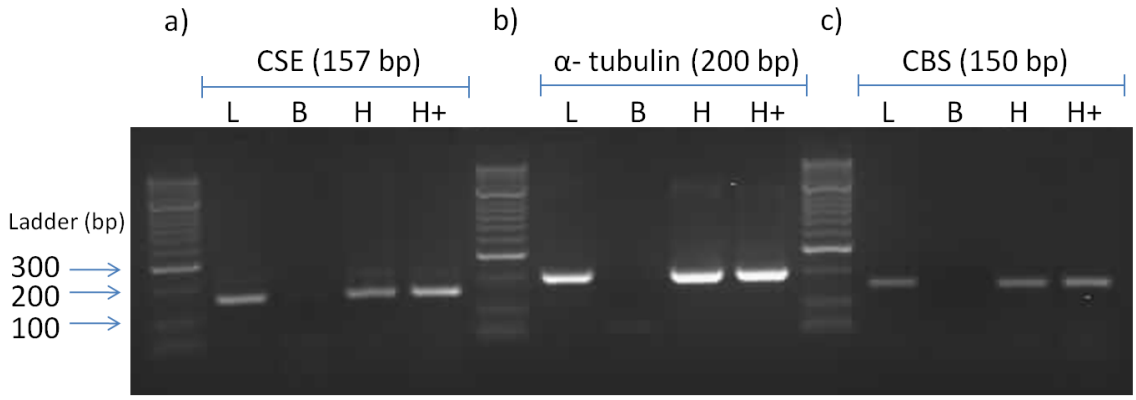

Figure 3.2.9. Image captured shows endpoint PCR expression for a) CSE (157 bp) b) α-tubulin loading control (200 bp) and c) CBS (150 bp). Human liver cDNA (L) was used as a positive control. Blank wells without a cDNA template (B) were used as a negative control. Both unstimulated HUVEC (H) and HUVEC stimulated with LPS (100 ng/ml) for 24 h (H+) express CSE, CBS and α-tubulin mRNA. Image is representative of 3 independent experiments.

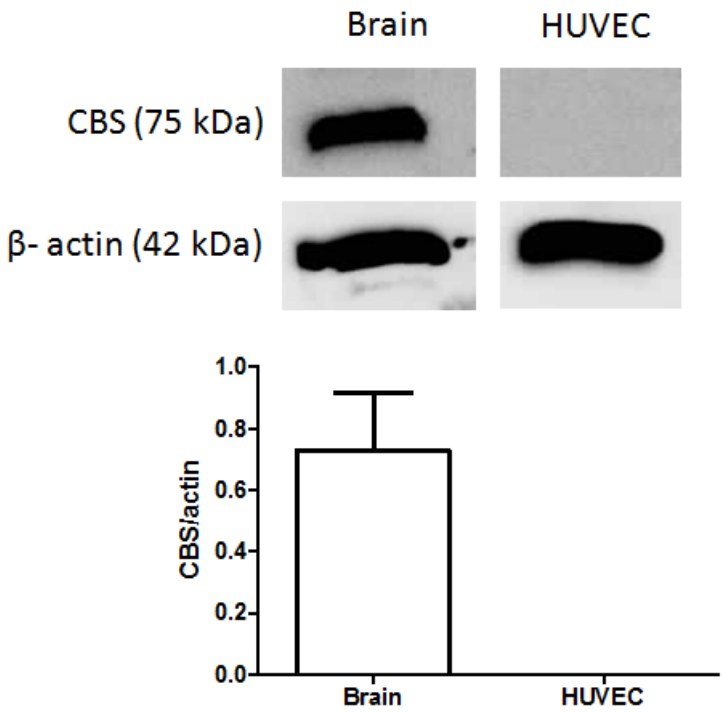

Figure 3.2.10. Western blot of murine brain tissue and HUVEC lysates probed for CBS protein. β-actin was used as the loading control. Data show representative western blots and a graph of showing densitometry of CBS standardised to β-actin loading control. Data show mean ± SEM, n = 4.

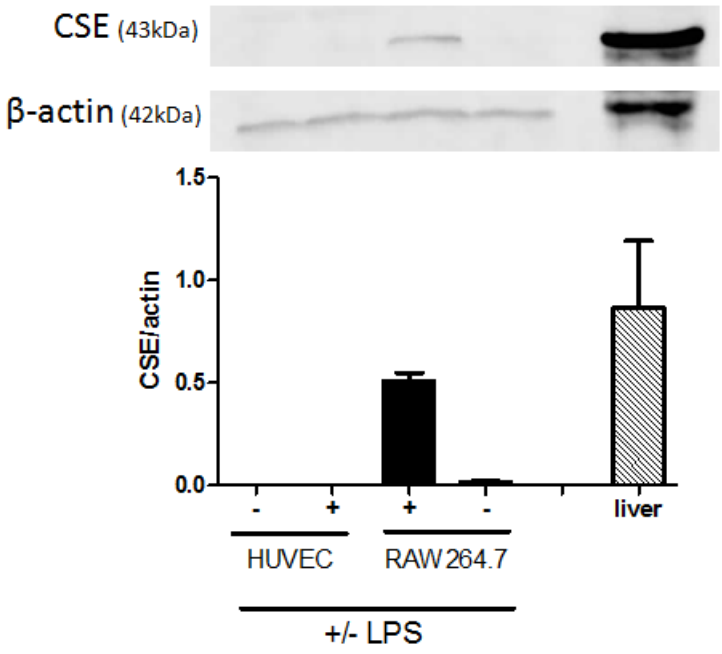

Figure 3.2.11. HUVEC and RAW264.7 cells were stimulated with LPS (100 ng/ml or 1 μg/ml) for 24 h. CSE protein was blotted with mouse liver as a positive control. Results show CSE densitometry values standardised to β-actin loading control, mean ± SEM, n = 4.

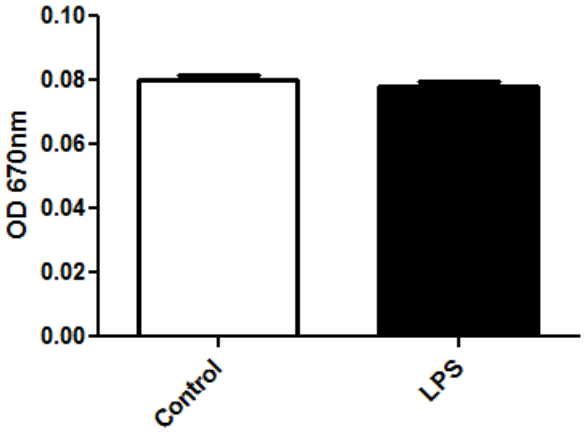

Figure 3.2.12. RAW264.7 cells were stimulated with LPS (1 µg/ml) for 24 h in SF-DMEM. At 24 h cell culture medium was assayed for H_2S using the methylene blue assay. Absolute values indicate 0 µM H_2S for all treatments. Results show mean optical density at 670 nm ± SEM, n = 3.

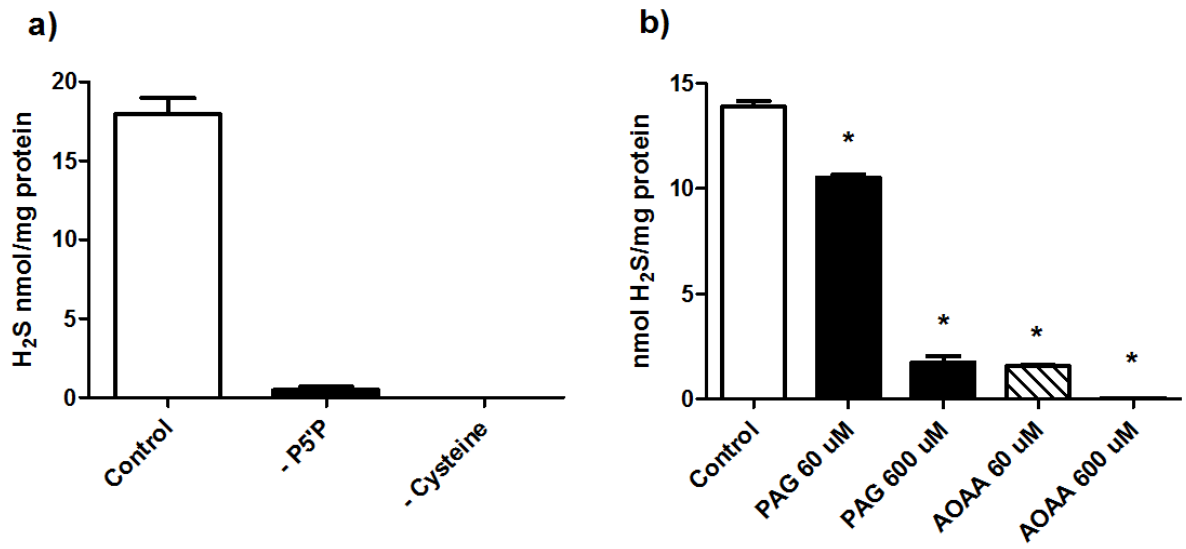

Figure 3.2.13. H_2S synthesising enzyme activity assay was carried out in rat liver homogenates, at 37^{o}C for 1 h. H_2S produced from homogenates was detected by the methylene blue colourimetric assay. Liver homogenates were incubated a) with both pyridoxal-5'-phosphate (P5'P) and L-cysteine (control), without P5'P (-P5'P) or without L-cysteine b) P5'P and L-cysteine ± CSE inhibitor DL-propargylglycine (PAG, 60 – 600 µM) or CBS inhibitor aminooxyacetic acid (AOAA, 60 – 600 µM). Results show mean ± SEM of H_2S nmol/mg protein, n = 4, *P<0.05 c.f. control.

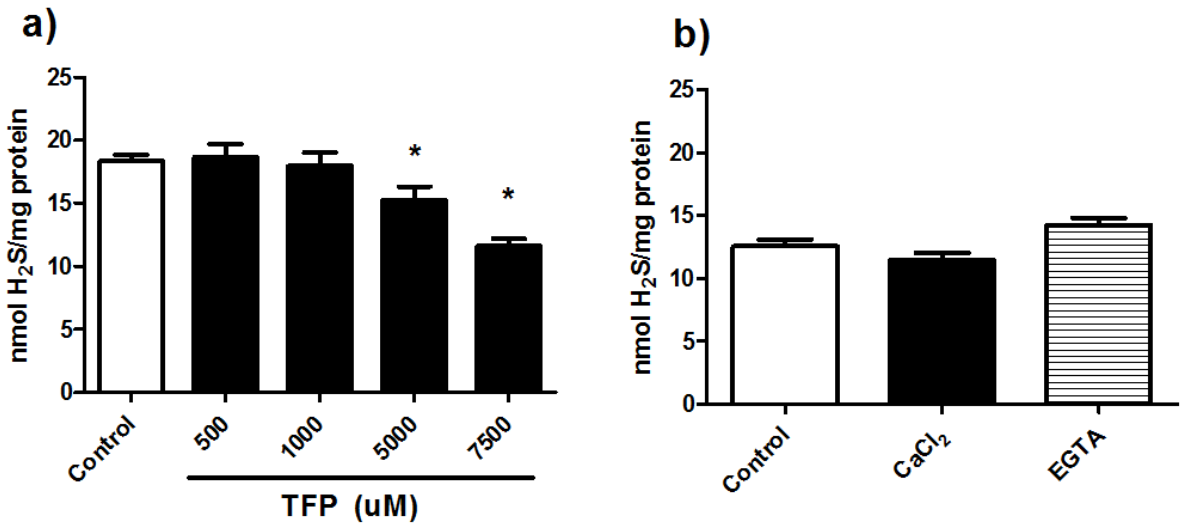

Figure 3.2.14. H_2S synthesising assay was carried out in rat liver homogenates incubated with a) Ca^{2+}/CaM antagonist trifluoperizine (TFP, 500 – 7500 µM) or b) $CaCl_2$ (500 µM) or EGTA (6000 µM), for 1 h at 37^{o}C. H_2S produced from liver homogenates was detected by the methylene blue colourimetric assay. Results show H_2S nmol/mg protein, mean ± SEM, n = 6 *P<0.05 c.f. control.

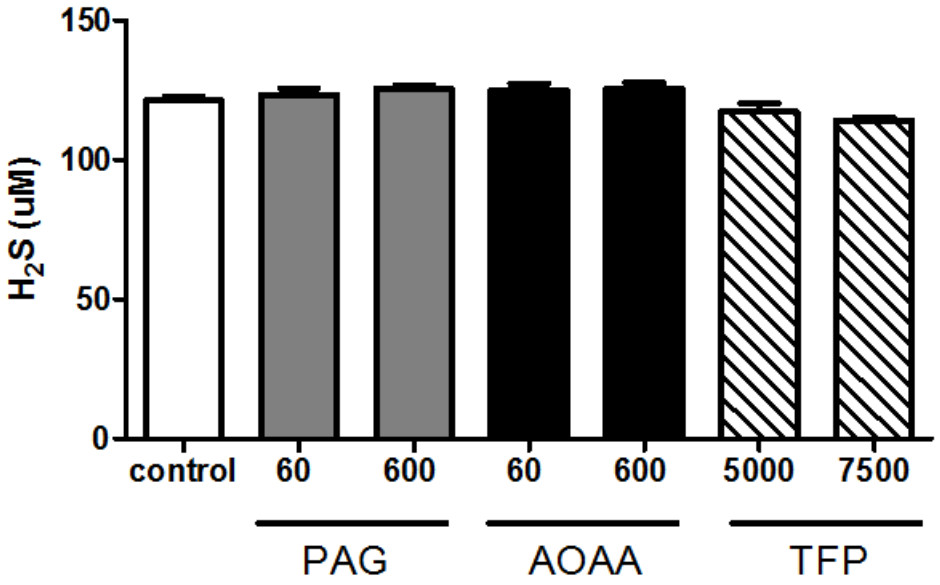

Figure 3.2.15. PAG (60 – 600 µM), AOAA (60 – 600 µM), TFP (5000 – 7500 µM) were incubated with NaHS (100 µM). Methylene blue colourimetric assay was carried out to ensure these inhibitors did not interrupt the colour formation. Results show H_2S µM, mean ± SEM, n = 3.

3.3. Discussion

The main findings from this chapter are:

1. H_2S is not detectable in the culture medium of A23187, MCh or BK stimulated endothelial cells (HUVEC and BAEC) or LPS stimulated RAW264.7 using the methylene blue colourimentric assay or the ISE
2. CSE/CBS mRNA, but not protein, is detectable in HUVEC
3. MCh does not induce a rise in intracellular Ca^{2+} in cultured endothelial cells
4. H_2S synthesising enzymes in the liver are unlikely to be Ca^{2+}/CaM dependent

It has previously been demonstrated that stimulating BAEC with A23187 (1 µM) or MCh (1 µM) results in a 1.5 and 3 fold increase in H_2S levels of the culture medium (Yang, et al. 2008). The reason for the discrepancy between the latter study and the current study is unclear as the identical ISE was used in both studies. The reason behind this difference is discussed.

3.3.1. Endothelial cell calcium response to A23187 and MCh

In the current study MCh (10 µM, 10 fold that of the latter study) did not induce a Ca^{2+} influx BAEC (Figure 3.2.4). A direct measurement of intracellular Ca^{2+} was not carried out in the latter study (Yang, et al. 2008), instead the assumption that H_2S release was mediated by a Ca^{2+} response was drawn from the response of A23187 and previous studies using MCh. Indeed, Ca^{2+} influx in endothelial cells on MCh/ACh stimulus have been noted in studies involving freshly isolated cells from coronary arteries of guinea pigs (Chen and Cheung 1992), rat thoracic aorta (Jen, et al. 2000), rabbit aorta (Wang

and van Breemen 1999), and arterioles from hamster cremaster muscles (Cohen and Jackson 2005). Interestingly, others have also failed to demonstrate a response from cultured cells treated with MCh (Loeb, et al. 1985, Tracey and Peach 1992). One study demonstrated the presence of muscarinic receptors only in freshly isolated endothelial cells but not cultured endothelial cells (Peach, et al. 1985). The reason behind the muscarinic receptor liability in cell culture conditions is unknown. However, the absence of muscarinic receptors in cultured endothelial cells is independent of species, vessel type and passage number (Peach, et al. 1985).

Interestingly, co-culturing smooth muscle cells with endothelial cells elicited a cGMP response from MCh (Loeb, et al. 1985) but the reason behind this is unknown. Whether the endothelial cells of previous studies (Yang, et al. 2008) were contaminated with smooth muscle cell remains unknown. Indeed, this may be one reason why the endothelial cells in their study contained CSE protein and elicited a response to MCh (Yang, et al. 2008).

In the current study, A23187 and BK were shown to increase intracellular Ca^{2+} in endothelial cells suggesting that the reason why H_2S was not detected in the medium of cultured cells was not due to the lack of response to these compounds.

3.3.2. H_2S quenching in the cell culture medium

Interestingly, H_2S standard curves made in cell culture medium containing high serum (20% v/v) concentrations significantly reduced the gradient of standard curve (Figure 3.2.5) suggesting a component in fetal calf serum (FCS) may be quenching or reacting with the H_2S rendering it undetectable by the methylene blue assay. Indeed, incubating NaHS with FCS, but not albumin, significantly quenched the H_2S as

demonstrated by both the methylene blue (Figure 3.2.6) and ISE (Figure 3.2.7). This result correlates with a finding from another group who demonstrated that FCS, but not bovine serum albumin, could absorb H_2S (Ishigami, et al. 2009). The cause of the H_2S quenching in serum is unknown and requires further investigation.

Stimulating lower passage HUVEC or BAEC with A23187 (1 µM) or BK (10 µM) in serum free conditions with or without L-cysteine pretreatment still did not lead to a measurable rise in H_2S determined by the methylene blue assay (Figure 3.2.8).

3.3.3. CSE expression in HUVEC and RAW246.7

The expression of CSE in endothelial cells has been slightly controversial: CSE protein has been described to be predominantly expressed in the endothelium lining of WT mice aorta (Yang, et al. 2008); whereas others have demonstrated that CSE protein is predominantly expressed in the media layer of the mouse aorta where the smooth muscle cells lye (Wang, et al. 2009b). Interestingly, CSE mRNA is not present in endothelial cells of rat aorta (Zhao, et al. 2001, Shibuya, et al. 2009a).

It has recently been demonstrated that HUVEC and BAEC express CSE protein (Yang, et al. 2008). In the current study, CSE mRNA but not protein was expressed in HUVEC. The reason why CSE protein was not detected in the current study is unclear and may be either a sensitivity issue or a difference in the antibodies utilised. Indeed, it is plausible there may be a difference in their own made CSE antibody and the commercially bought CSE antibody utilised in this study. It is possible that the lack of CSE protein detected in HUVEC is due to poor commercial antibodies against human CSE. The presence of CSE and CBS message in HUVEC of the current study suggests that CSE protein is likely to be expressed in human endothelial cells. Indeed, H_2S

synthesising enzyme activity appears to be present in BAEC and HUVEC (Table 3.2.1). However, the localisation of CAT and 3-MST has recently been described in rat thoracic aorta endothelial cells (Shibuya, et al. 2009a), and may be another source of H_2S in endothelial cells. Indeed it will be interesting to take this study further to determine the presence of CAT and 3-MST in HUVEC.

LPS has been shown to stimulate CSE expression in RAW 246.7 macrophages (Zhu, et al. 2010), as well as in the tissues of LPS treated mice (Li, et al. 2005). Although in the present study CSE protein was not detected in basal and LPS stimulated HUVEC, an increase in CSE protein levels were detected in LPS stimulated RAW246.7 cells (Figure 3.2.11). However, H_2S was not detected in the culture medium of LPS stimulated RAW246.7 at 24 h even though CSE protein was strongly expressed at this time point (Figure 3.2.12) suggesting that the methods used in this study to detect H_2S levels may not be sensitive enough to detect small changes in H_2S production.

3.3.4. Methods used to detect H_2S

There is much debate regarding the artificial elevation of sulfide in methods used to measure H_2S (Olson 2009). Results presented in this report suggest the methods are insensitive to low levels of H_2S produced by cells. Much of the debate regarding the artificial elevation of sulfide relates to the measurement of plasma H_2S. However, this has provided clues as to why there may be differences in the H_2S detected in the culture medium of the current work and in previous studies (Yang, et al. 2008).

Speculations have arisen among the accuracy of the methods used to detect H_2S, due the extreme pH conditions found in the conventional H_2S detection assays which may induce the release of sulfur from protein leading to an artificial rise in the

sulfide detected (Olson 2009). Other than H_2S, defined as free total free sulfide (H_2S, HS^- and S^{2-}) in the context of this thesis, there are other forms of endogenous sulfur in the body such as sulfane sulfur. Sulfane sulfur atoms are reactive sulfur atoms with an unusual valency or oxidation state of 0 or -1 (in contrast to sulfide with a valency of -2) and are covalently bound to other sulfur atoms (Westley, et al. 1983, Toohey 1989, Iciek and Wlodek 2001). Some compounds that contain sulfane sulfur atoms include thiosulfate, disulfides, polysulfides and elemental sulfur (Westley, et al. 1983). Compounds containing sulfane sulfur have antioxidative properties and have biological function such as the regulation of enzyme activity (Iciek and Wlodek 2001). These compounds containing reactive sulfane sulfur atoms can be endogenously produced during anaerobic cysteine desulfuration (Westley, et al. 1983) by enzymes such as CSE, 3-MST (3-mercaptopyruvate sulfurtransferase), CAT (cysteine transaminase) and rhodanese (Iciek and Wlodek 2001). Sulfane sulfur is also present on plasma albumins, which is hypothesised as a carrier for the transport of sulfane sulfur to the rest of the body (Westley, et al. 1983).

Sulfane sulfur is highly reactive and can be released from the parent molecule in the form of sulfide (Iciek and Wlodek 2001). Furthermore, sulfane sulfur is liable for release by a number of different conditions e.g. excess glutathionine (GSH) or presence of dithiothreitol (DTT) (Warenycia, et al. 1990, Ishigami, et al. 2009). Indeed, some authors have suggested the physiological release of H_2S from bound sulfane sulfur by physiological levels of GSH and cysteine in the neurons of rodents (Ishigami, et al. 2009). These authors also suggested that free H_2S may be stored as bound sulfur for later release (Ishigami, et al. 2009). More importantly, in the context of this chapter, the alkaline antioxidant buffer associated with the ISE, used to convert all sulfide to S_2^-,

has been suggested to induce protein desulfuration (Olson 2009). This effect has also been noted by others (Khan, et al. 1980, Whitfield, et al. 2008). One group demonstrated that when 5% bovine serum albumin was placed in antioxidant buffer, there was a measurable rise in sulfide detected by the ISE which continued to increase over 3 h (Whitfield, et al. 2008). Indeed, in the current study it was noted that making H_2S standard curves in cell culture medium for the ISE lead to a continuous 'drift' or change in baseline voltage over time, or what Whitfield describes as the release of H_2S from albumin, making it difficult to derive an accurate absolute value for varying concentrations of added sulfide. As a result, percentage change rather than absolute sulfide values were used when measuring sulfide values with the ISE in A23187 stimulated HUVEC (Figure 3.2.2). Interestingly, incubating this sulfide probe in a range of different substances in the absence of sulfide, caused a measurable change in potential difference (Figure 3.2.7). Indeed, this probe detected 'sulfide' (a measurable reduction in voltage) on baseline incubation with $MgCl_2$, $CaCl_2$, $FeCl_2$, blood, albumin, fetal calf serum (FCS) alone without added sulfide. Although, potentially sulfide may be released from bound sulfur from proteins in blood, albumin or FCS, no obvious protein was present in solutions of $MgCl_2$, $CaCl_2$ or $FeCl_2$. It brings up some speculation of whether the apparently selective sulfide probe is measuring a change in sulfide, or whether background changes also induce changes in potential difference. Extreme pH < 5.4 has also shown to release sulfide from acid-liable sulfur sources predominately from the iron-sulfur centers in mitochondrial enzymes (Ubuka, et al. 2001, Ishigami, et al. 2009). Due to the acidic solution used in the methylene blue colourimetric method, this assay may be associated with an artificial rise in sulfide from potential acid-liable sulfur sources in plasma or tissues (Olson 2009).

It has previously been shown that baseline sulfide levels are increased within 10 min of stimulating BAEC with A23187 and MCh using the ISE H_2S detection method (Yang, et al. 2008). Although these methods may lead to an artificial elevation of sulfide, sulfide levels were not detected in both basal as well as A23187 stimulated cells (HUVEC and BAEC) using both the methylene blue method and the ISE. In this study the ISE could only convincingly detect sulfide levels above 10 µM H_2S, whereas the methylene blue method appeared to be the more sensitive method with detection levels of ~3 µM H_2S. Although a 3 fold rise in H_2S was detected in MCh stimulated BAEC (Yang, et al. 2008) absolute H_2S concentrations and the sensitivity of the ISE were not stated.

The difference between this study and the latter study (Yang, et al. 2008) brings up some important questions: 1. Whether the ISE in the latter study was indeed measuring sulfide; 2. Whether MCh in their study can induce a rise in intracellular Ca^{2+} in endothelial cells? If not, whether the rise in H_2S detected by the ISE was due to a non-selective effect of MCh or the non-specific effect of the ISE? If MCh does indeed induce Ca^{2+} rise, could it be due to presence of smooth muscle cell contamination in the endothelial cell culture? (Loeb, et al. 1985). If there is smooth muscle cell contamination in the endothelial cell culture, could this be the reason for the presence of CSE protein expression in the latter study?

3.3.5. H_2S enzyme activity assay in liver homogenates ± Ca^{2+}/CaM antagonists

Liver is source of the H_2S synthesising enzymes, CSE and CBS, and in the presence of substrate (L-cysteine) and cofactors (P5'P), detectable amounts of H_2S are produced (Li, et al. 2005, Kabil, et al. 2011). In the current study, measurable amounts of H_2S

were detected from rat liver tissue on incubation with L-cysteine and P5'P, and this effect was inhibited by irreversible CSE inhibitor PAG and CBS inhibitor AOAA (Figure 3.2.13). PAG has a reported IC_{50} ~55 µM (Mok, et al. 2004) in rat liver homogenates. In the current study, PAG (60 µM) inhibited ~ 24.17% ± 1.10 H_2S nmol/mg protein, at the same concentration AOAA (60 µM) CBS inhibitor inhibited ~88.73 ± 0.46 H_2S nmol/mg protein of control. In neuronal cells, AOAA has an IC_{50} ~ 31 µM (Rzeski, et al. 2005). The greater reported potency of AOAA to PAG correlates with the current data that show AOAA has a greater inhibitory effect than PAG at the same drug concentration in the liver homogenates.

To test whether H_2S synthesising enzymes were Ca^{2+}/CaM dependent, an H_2S synthesising enzyme assay was carried out using liver homogenates in the presence of a Ca^{2+}/CaM antagonist trifluoperizine (TFP). TFP works by binding to Ca^{2+}-bound calmodulin to induce a conformational change so Ca^{2+}/CaM can no-longer interact with target molecules (Vandonselaar, et al. 1994). High concentrations of TFP 5 mM were required to significantly inhibit the production of H_2S in the enzyme assay (Figure 3.2.14). The effects of TFP, PAG and AOAA were not as a result of inhibiting the biochemical colour change associated with the methylene blue colourimetric assay (Figure 3.2.15). The concentrations of TFP used in this study to block H_2S synthesis in liver were much higher than the concentrations of TFP used in the literature to block Ca^{2+}/CaM action. For example, TFP inhibited the $^{\bullet}NO$-evoked cGMP response in rat cerebellar cells with an EC_{50} 180 µM (James, et al. 2009). In the cytosolic fraction of rat liver homogenates TFP 25 µM inhibited the activity of phosphocholine cytidylyltransferase an enzyme involved in phosphatidylcholine biosynthesis by 5-fold (Pelech, et al. 1983). TFP is a competitive antagonist and higher concentrations of drug

may be necessary to block actions of these enzymes in the presence of excess Ca^{2+}. However, due to the large concentrations of TFP used in this study it is plausible that inhibitory effect on H_2S enzyme activity is a non-selective effect of TFP. Certainly in the current study another Ca^{2+}/CaM antagonist is required to confirm the Ca^{2+}/CaM dependence of H_2S enzyme activity in liver homogenates and to confirm whether the inhibitory effects of TFP are as a result of a non-selective effect of this compound.

In the current study, the addition of Ca^{2+} (500 μM) did not significantly increase H_2S enzyme activity in liver homogenates (Figure 3.2.14). This suggests that the activity of H_2S enzymes are either independent of Ca^{2+} or, excess Ca^{2+} is already present in liver homogenates and adding more may not induce further effect. Interestingly in the current study addition of Ca^{2+} chelator EGTA (6 mM) did not significantly reduce H_2S enzyme activity (Figure 3.2.14). However, one previous study showed that the addition of both Ca^{2+} (2 mM) and CaM (5 μM) increased the activity of purified CSE by two fold (Yang, et al. 2008). In the current study, only Ca^{2+} was added to the liver homogenates, further studies in the presence of both added Ca^{2+} and CaM may be necessary to determine the Ca^{2+}/CaM dependence of H_2S enzymes in liver homogenates. Earlier studies using purified CSE (Chiku, et al. 2009) and CBS (Kraus, et al. 1978, Kraus and Rosenberg 1983) have shown that these enzymes did not require added Ca^{2+} to induce its activity. However, these latter studies did not add Ca^{2+}/CaM to determine whether a further increase in enzyme activity could be achieved. Further studies using purified CSE enzymes with added Ca^{2+}/CaM are necessary to confirm previous findings (Yang, et al. 2008).

3.3.6. CSE, H_2S and endogenous blood pressure regulation

Earlier studies (Yang, et al. 2008) have demonstrated that $CSE^{-/-}$ KO mice develop an age dependent hypertension as a result of endothelial cell dysfunction which was suggested to be as a result of depleting CSE protein in the endothelium. On further examination, these authors demonstrated that both HUVEC and BAEC contain CSE protein, and stimulating BAEC with A23187 (1 μM) or MCh (10 μM) induced the release of measurable amounts of H_2S in the cell culture medium within 10 min (Yang, et al. 2008). This suggested that stimulating endothelial cells with agents that cause a rise in intracellular Ca^{2+} will lead to the activation of CSE and the production of H_2S (Yang, et al. 2008). Exogenous H_2S has been shown by various laboratories to induce vascular relaxation (Zhao, et al. 2001, Ali, et al. 2006), and as a result these authors (Yang, et al. 2008) suggested that the endothelial cell dysfunction and age dependent hypertension observed in the $CSE^{-/-}$ KO mice was due to a lack of H_2S release from the endothelium.

Plasma concentrations of H_2S have been reported to range from 2 μM (Olson 2009) to 280 μM (Han, et al. 2006), with around average reported plasma concentrations ~ 40 μM H_2S using the typical methods of H_2S detection that is the methylene blue assay and the ISE (Olson 2009). Interestingly, the use of a sensitive polorgraphic sensor which can measure H_2S (up to 100 nM sulfide) in real time without the need for chemical modification, could not detect H_2S in the plasma in a number of animal species including mouse, rat, trout, pig, lamprey and cow (Whitfield, et al. 2008). Moreover, injection of H_2S donors into animals only results in a transient (<30 sec) reduction in blood pressure (Zhao, et al. 2001), which may suggest that the level of free H_2S in blood plasma is transient. In a human phase I safety study, a significant rise in blood sulfide (42.5 ± 8.9 c.f. 106.2 ± 54.1 μM H_2S, *P = 0.0004) was described on

an intravenous infusion of Na_2S (i.v. 0.20 mg kg−1 over a period of 1 min) (Toombs, et al. 2010). However, this rise in blood sulfide did not lead to a change in blood pressure (Toombs, et al. 2010). This may suggest that >100 µM plasma H_2S is required to a vasorelaxant effect. The current consensus, is that plasma concentration of endogenous free sulfide <100 nM (Whitfield, et al. 2008) is far less than the predicted concentration of H_2S necessary to induce a vasorelaxant effect (>100 µM H_2S *ex vivo*). Moreover, low concentrations of H_2S (<100 µM) have been described to have a vasoconstrictive effect in *ex vivo* blood vessels (Ali, et al. 2006). Furthermore, a distinct smell of rotten eggs would be expected if concentrations of 40 µM free H_2S in plasma were present. This raises questions whether micromolar H_2S plasma are present physiologically and whether endogenous free H_2S induces vasodilatation. It is also interestingly to note whilst the $CSE^{-/-}$ KO mice in the latter study demonstrated age dependent hypertension (Yang, et al. 2008), another laboratory who also developed $CSE^{-/-}$ KO did not observe the same phenomenon (Ishii, et al. 2010). The reason behind the differences in the $CSE^{-/-}$ KO mice is unclear and evidently requires further examining.

However, on another note, although H_2S may exist only transiently in the blood, it is possible that it can induce its effects as an endogenous signalling molecule by exerting its effects within cells or between adjacent cells. However, its role as a circulating endogenous signalling molecule is uncertain.

3.3.7. Conclusion

Overall, due to the limitation in the sensitivity and selectivity of the methods used to measure H_2S release *de novo* in culture, there is insufficient evidence to provide a definitive conclusion as to whether H_2S is an endothelial cell derived relaxing factor. It is questionable whether H_2S synthesising enzymes found in liver are Ca^{2+}/CaM dependent, further studies are needed to confirm this. The role of endogenous H_2S in cardiovascular physiology is still unclear and is limited by lack of reliable methods used to measure and detect endogenous H_2S generation.

CHAPTER 4: Cross talk between Cystathionine Gamma Lyase and Nitric Oxide Synthase

4. Introduction

It has been suggested that a potential crosstalk exists between $^{\bullet}NO$ and H_2S. H_2S donors attenuate iNOS expression and $^{\bullet}NO$ production in microglia (Hu, et al. 2007) and RAW264.7 cells (Oh, et al. 2006) exposed to LPS. Conversely, NaHS (25 – 100 μM) has also been shown to increase IL-1β induced iNOS protein expression in rat vascular smooth muscle cells (Jeong, et al. 2006). NaHS (50-100 μM) has been reported to reduce eNOS phosphorylation and eNOS maximal activity in HUVEC (Geng, et al. 2007). In contrast to the latter study, in a model of ischemia reperfusion, continuous infusion of NaHS (100 μM postconditioning, 2 min continuous infusion) was shown to stimulate PKC and Akt and induce the phosphorylation of eNOS in cardiomyocytes (Yong, et al. 2008).

In homogenates of aortic tissue, administration of the $^{\bullet}NO$ donor SNP concentration-dependently increases H_2S synthesis suggesting that $^{\bullet}NO$ may increase CSE activity (Zhao, et al. 2001). Furthermore, in the same study the $^{\bullet}NO$ donor SNAP (*S*-nitroso-*N*-Acetyl-D,L-Penicillamine) increased CSE expression in cultured vascular smooth muscle cells (Zhao, et al. 2001). In a model of ischemia-reperfusion in the rat kidney, CBS activity was inhibited (Prathapasinghe, et al. 2008). However, this effect was reversed on injecting rats with a $^{\bullet}NO$ scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) suggesting $^{\bullet}NO$ metabolites may contribute to the inhibition of CBS activity in this model (Prathapasinghe, et al. 2008).

Other than “gas-enzyme” regulation, a “gas-gas” interaction has also been proposed. It has been suggested that $^{\bullet}NO$ and H_2S may chemically react to form a

novel nitrosothiol (RSNO) *in vitro* (Whiteman, et al. 2006). In support of this, incubating SNP (100 µM) with NaHS (100 µM) did not elevate cGMP levels in cultured RAW264.7 cells, in contrast to SNP alone (Whiteman, et al. 2006). Moreover, in anaesthetised rats SNP induced a significant reduction in mean arterial blood pressure, whereas co-administration of SNP (16.5 nmol kg−1, i.v) and NaHS (5 µmol/kg, i.v) abolished this reduction in blood pressure (Ali, et al. 2006). H_2S can also react with peroxinitrite ($ONOO^-$) to form a novel thionitrate ($HSNO_2$) that is capable of releasing $^{\bullet}NO$ (Filipovic, et al. 2011).

$^{\bullet}NO$ and H_2S have very similar biological properties and exert similar effects within the vascular system. They are both small molecule gases capable of diffusing through cell membranes and are endogenously produced by endothelial cells and/or smooth muscle cells in blood vessels (Wang 2002a). $^{\bullet}NO$ and H_2S also exert anti-hypertensive effects: either through the activation of soluble guanylate cyclase and subsequent elevation of 3',5'-cyclic guanosine monophosphate (cGMP) (Lincoln, et al. 2001); or through direct activation of K_{ATP} channels on VSMC to induce hyperpolarisation, respectively (Zhao, et al. 2001). H_2S may also potentially enhance the effects of $^{\bullet}NO$ through inhibition of phophodiesterase activity and subsequent elevation of cGMP levels (Bucci, et al. 2010).

Under inflammatory conditions, both $^{\bullet}NO$ donors (Kobayashi 2010) and H_2S donors (Li and Moore 2008) have been shown to exert both pro- and anti-inflammatory effects. Moreover, both $^{\bullet}NO$ (Napoli, et al. 2006) and H_2S (Wang, et al. 2009b) inhibit the development of atherosclerotic plaque formation. Indeed, both $^{\bullet}NO$ and H_2S have very similar actions and it is possible that loss of signalling from one pathway may lead to a compensatory effect of the other.

Hypothesis: H_2S synthesising enzymes will compensate for the loss of NOS activity.

Thus, the aim of this study was to:

1. Compare the H_2S synthesising enzyme activity in multiple organs from wildtype (WT), $eNOS^{-/-}$ and $iNOS^{-/-}$ KO mice
2. Compare protein expression of both CSE or CBS in organs from WT and $eNOS^{-/-}$ KO mice.

4.1. Materials and Methods

4.1.1. Animal housing and breeding

Male eNOS$^{-/-}$ and iNOS$^{-/-}$ (30 g) were a kind gift from Dr. Adrian Hobbs (University College London, UCL). Both eNOS$^{-/-}$ and iNOS$^{-/-}$ mice were bred on a C57BL/6 background. In all studies, age-gender matched wildtype (WT) littermates were used as controls. These mice were housed in the UCL Biological Services Unit (Euston) in a climatically controlled environment (20^{o}C, 12 h light cycle). Laboratory chow and drinking water were available *ad libitum*. All experiments were carried out blinded and according to the 1986 UK Home Office Animals Scientific Procedures Act.

4.1.2. Tissue processing

Mice were anaethesised under isoflurane (4% v/v) before cardiac puncture was carried out using heparinised syringes. The mice were then killed via cervical dislocation and organs removed and flash frozen in liquid nitrogen prior to storage at -80^{o}C.

4.1.3. H_2S synthesising activity

Whole mouse tissue was homogenised in $KHPO_4$ (100 mM, pH 7.4) as follows: liver (5% w/v), kidney (10% w/v) and brain, lung, spleen, stomach and small intestine (15% w/v). H_2S synthesising activity measured as previously described (Methods 2.11).

Western blot for CSE and CBS in mouse tissues

Mouse tissues were lysed in 10% w/v (with the acceptation of aorta and brain which were lysed at 5% w/v and 20% w/v respectively) cell lysis buffer (Method 2.7) using a Polytron Homogeniser. Cell lysates were then left on ice for 2 h before centrifuging at 13,000 rpm, 4^{o}C for 10 min. The supernatant was collected and re-centrifuged at 13,000 rpm, 4^{o}C for 10 min, to remove excess debris. The clear supernatant was aliquoted and frozen at -20^{o}C for Western blotting (Method 2.7). Immunoreactive bands at 42 kDa and 70 kDa corresponding to CSE and CBS respectively, were quantified with a Syngene gel quantification system. Due to problems with stripping CSE antibody from the membrane, the loading control (β-actin) was analysed on a separate Western blot from the same sample run in parallel with the CSE/CBS blot.

4.2. Results

4.2.1. H_2S synthesising activity and CSE/CBS protein expression in WT mice

H_2S synthesising activity and CSE/CBS protein expression were initially assessed in multiple organs from WT mice. Clear differences in H_2S production were observed in the different tissues under investigation. The organs with the greatest H_2S synthesising enzyme activity (H_2S nmol/mg protein) were liver (10.08 ± 0.76) > kidney (3.93 ± 0.28) > small intestine (2.40 ± 0.21) > stomach (0.95 ± 0.15) > brain (0.56 ± 0.02) (Figure 4.2.1 a). H_2S enzyme activity was at the limit of detection in lung (0.01 ± 0.01) and spleen (0.01 ± 0.03) tissues (Figure 4.2.1 a).

CSE protein expression appeared to correlate with H_2S enzyme activity assay (Figure 4.2.1 b). CSE protein was highest in liver > kidney > small intestine > stomach but undetectable in brain, lung, spleen and heart tissue (Figure 4.2.1 b).

CBS protein expression was predominantly found in the brain with some CBS protein found in lung tissue (Figure 4.2.1 c) and did not correlate with the H_2S enzyme activity assay (Figure 4.2.1 a and 4.2.1 c) suggesting that the H_2S enzyme assay may be measuring CSE activity alone, rather than both CSE and CBS activity.

4.2.2. H_2S synthesising enzyme activity in iNOS$^{-/-}$ mice

To determine whether H_2S enzyme activity and expression were altered in a system which lacks NOS expression and activity, the above experiments were repeated using iNOS$^{-/-}$ mice. There was no difference in H_2S synthesising enzyme activity in most of the organs (brain, lung, liver, spleen, stomach and small intestine) of the iNOS$^{-/-}$ mice

compared to WT (Figure 4.2.2 – 4.2.8). Interestingly, there was a significant reduction in H_2S enzyme activity in kidney tissue of $iNOS^{-/-}$ c.f. WT (Figure 4.2.3, $P<0.05$).

4.2.3. CSE and CBS protein expression and H_2S synthesising activity in $eNOS^{-/-}$ mice

H_2S synthesising activity and CSE protein expression were also examined in tissues of $eNOS^{-/-}$ mice. In liver tissue, a significant ($P<0.05$) increase in H_2S synthesising enzyme activity was measured in $eNOS^{-/-}$ compared to WT (Figure 4.2.2 a) and this correlated with an increased protein expression for CSE (Figure 4.2.2 b). A significant ($P<0.05$) increase in H_2S enzyme activity in the stomach (Figure 4.2.4 a) and small intestine (Figure 4.2.5 a) was observed in $eNOS^{-/-}$ compared to WT. However, this did not correlate with a change in CSE protein expression in stomach and small intestine of $eNOS^{-/-}$ (Figure 4.2.4 b and 4.2.5 b).

There was no significant difference in H_2S enzyme activity and/or CSE protein expression of $eNOS^{-/-}$ and WT mice in: kidney tissue (Figure 4.2.3), brain (Figure 4.2.6), lung (Figure 4.2.7), spleen (Figure 4.2.8), heart and aorta (Figure 4.2.9).

Lung and brain tissue contain basal amounts of CBS protein (Figure 4.2.1 c), therefore western blots for CBS protein in WT and $eNOS^{-/-}$ lung and brain tissue were carried out. No significant difference in CBS protein expression was found in brain or lung tissue of $eNOS^{-/-}$ KO compared to WT (Figure 4.2.6 c and 4.2.7 c).

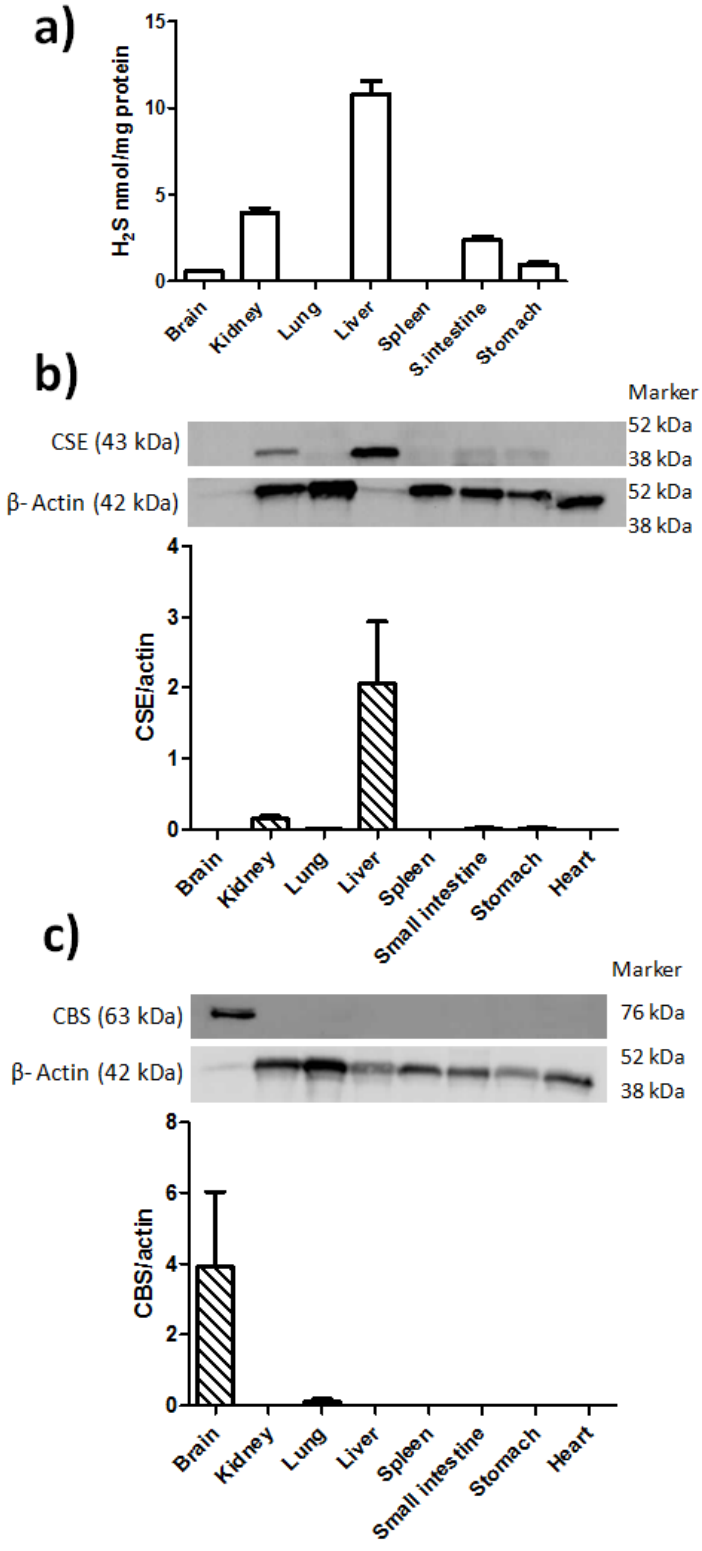

Figure 4.2.1. WT mouse organs a) H_2S synthesising enzyme activity where data is expressed as H_2S produced (nmol/mg protein), b) Representative western blots of CSE and β-actin loading control. Graph shows densitometry values of CSE standardised to β-actin c) Representative western blot of CBS and β-actin loading control. Graph shows densitometry values of CBS standardised to β-actin. All data are shown as mean ± SEM, n = 6.

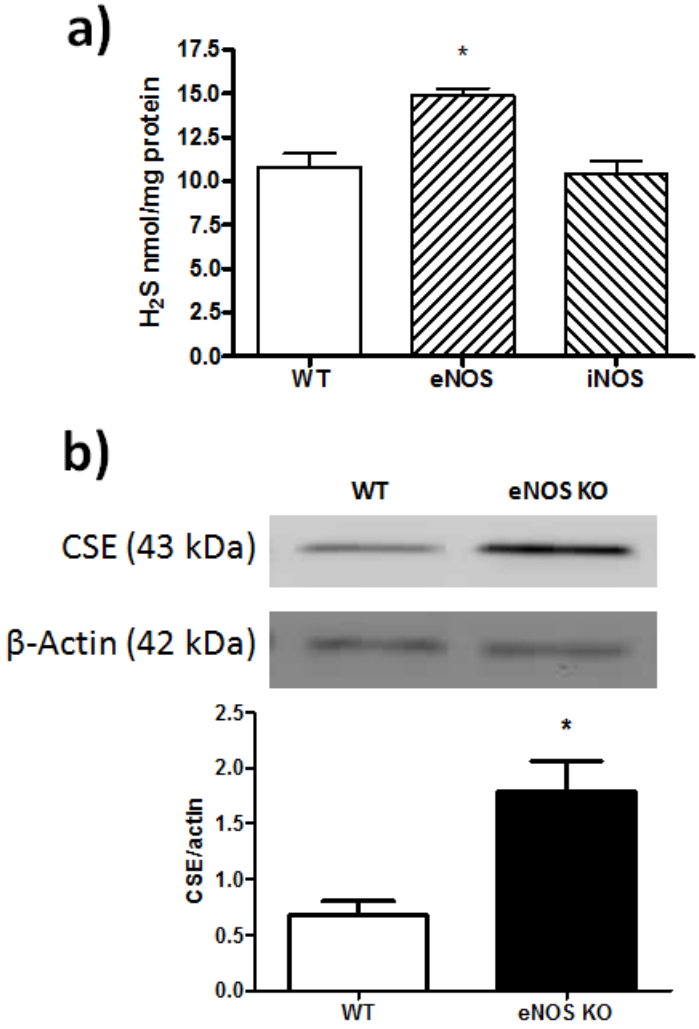

Figure 4.2.2. Graph shows liver tissue a) H_2S synthesising enzyme activity as H_2S nmol/mg protein from $eNOS^{-/-}$, $iNOS^{-/-}$ and WT b) representative Western blot for CSE and β-actin (5 µg liver tissue, $eNOS^{-/-}$ c.f. WT) with graphs representing densitometry values of CSE standardised to β-actin. All data are shown as mean ± SEM, n = 6, *P<0.05 c.f. WT.

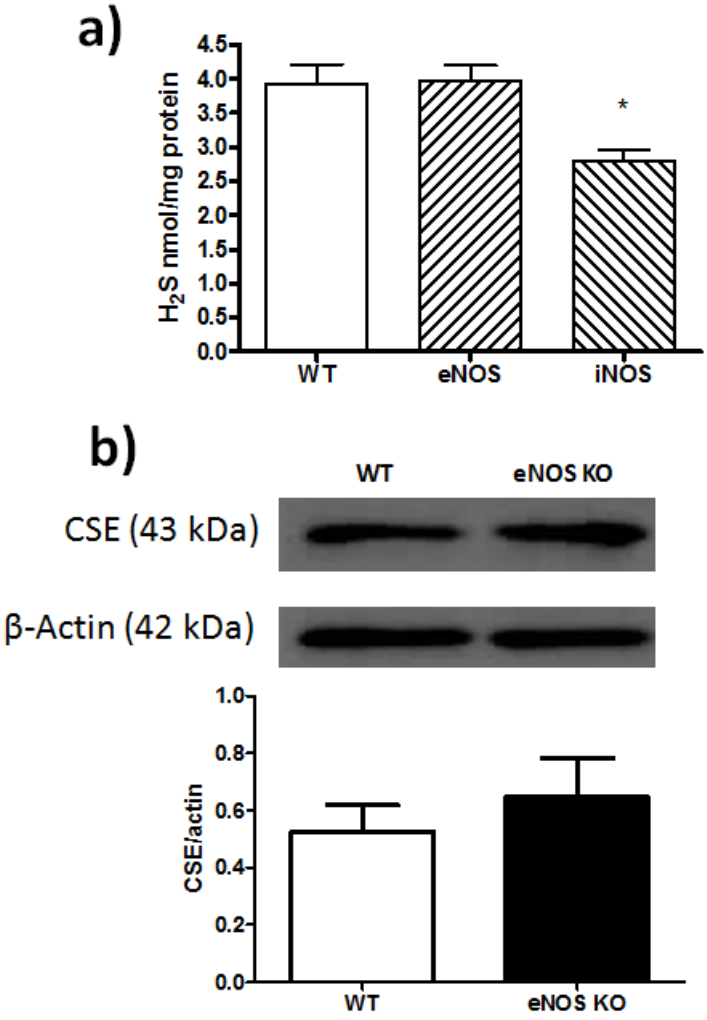

Figure 4.2.3. Graph shows kidney tissue a) H_2S synthesising enzyme activity (H_2S nmol/mg protein) from $eNOS^{-/-}$, $iNOS^{-/-}$ and WT b) representative Western blot for CSE and β-actin from $eNOS^{-/-}$ and WT kidney tissue (5 µg) with graphs representing densitometry values of CSE standardised to β-actin. All data are shown as mean ± SEM, n = 6, *P<0.05 c.f. WT.

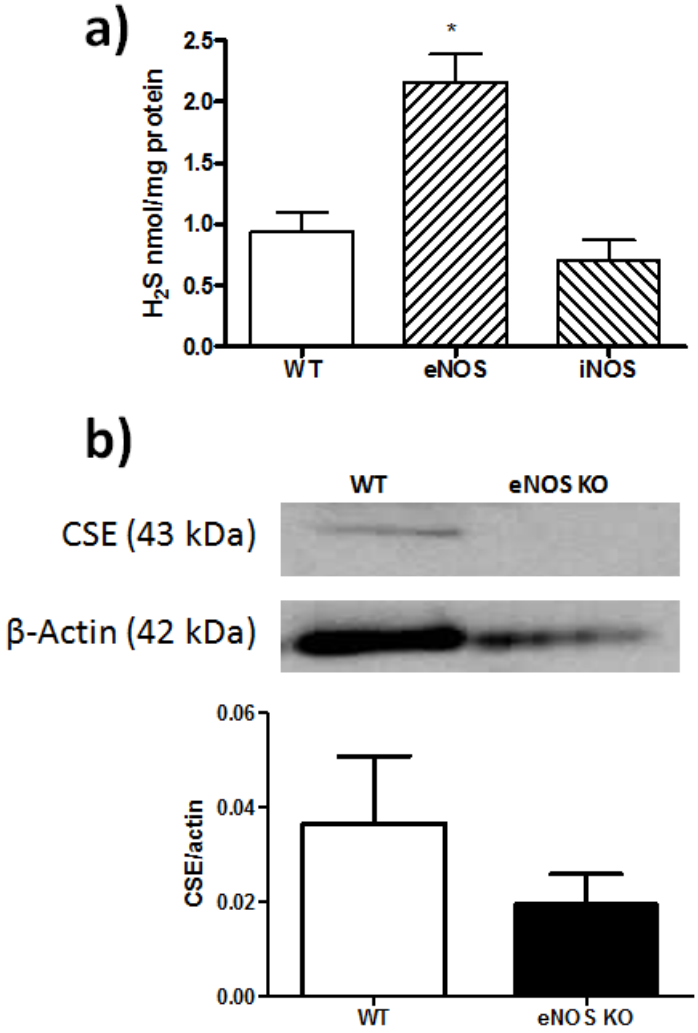

Figure 4.2.4. Stomach tissue a) H_2S synthesising enzyme activity in $eNOS^{-/-}$, $iNOS^{-/-}$ and WT (H_2S nmol/mg protein) b) representative CSE and β-actin Western blot from $eNOS^{-/-}$ and WT stomach tissue (50 µg). Graph below Western blot shows densitometry values of CSE standardised to β-actin. All data are shown as mean ± SEM, n = 6, *P<0.05 c.f. WT.

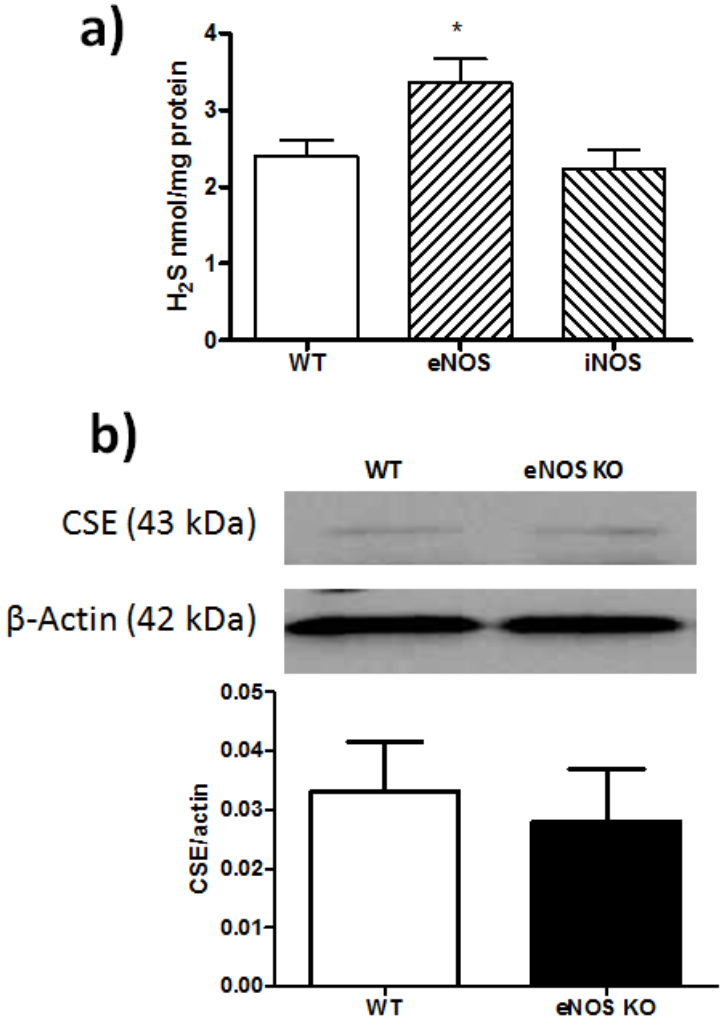

Figure 4.2.5. Data show a) H_2S enzyme activity from small intestine of WT, $eNOS^{-/-}$ KO and $iNOS^{-/-}$ KO mice represented as H_2S ng/mg protein b) representative western blot of CSE and β-actin from $eNOS^{-/-}$ and WT small intestine tissue (50 µg). Graph below Western blot shows densitometry values of CSE standardised to β-actin. All data are shown as mean ± SEM, n = 6, *P<0.05 c.f. WT.

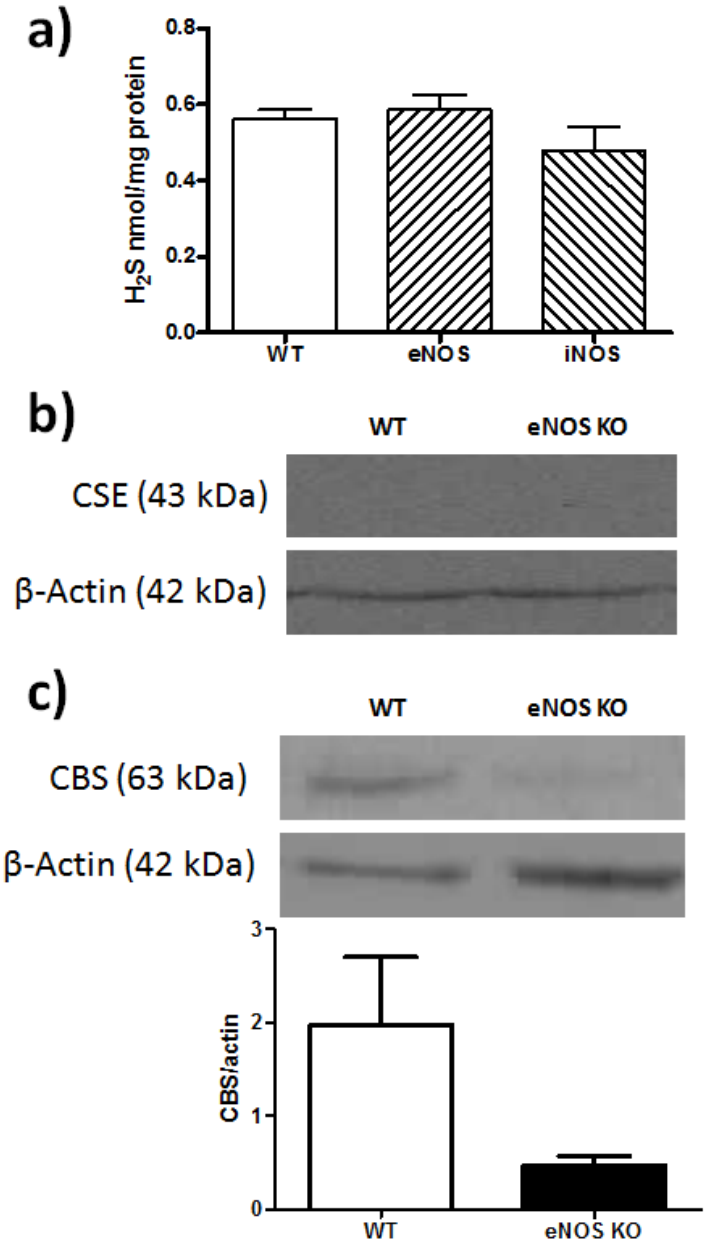

Figure 4.2.6. Graph shows brain a) H_2S enzyme activity (H_2S nmol/mg protein) from WT, $eNOS^{-/-}$ and $iNOS^{-/-}$ mice b) representative western blot of CSE and β-actin loading control from $eNOS^{-/-}$ and WT brain tissue (40 µg) c) representative western blot of CBS and β-actin loading control with graphs representing densitometry values of CBS standardised to β-actin from $eNOS^{-/-}$ and WT brain tissue (40 µg). All data are shown as mean ± SEM, n = 6.

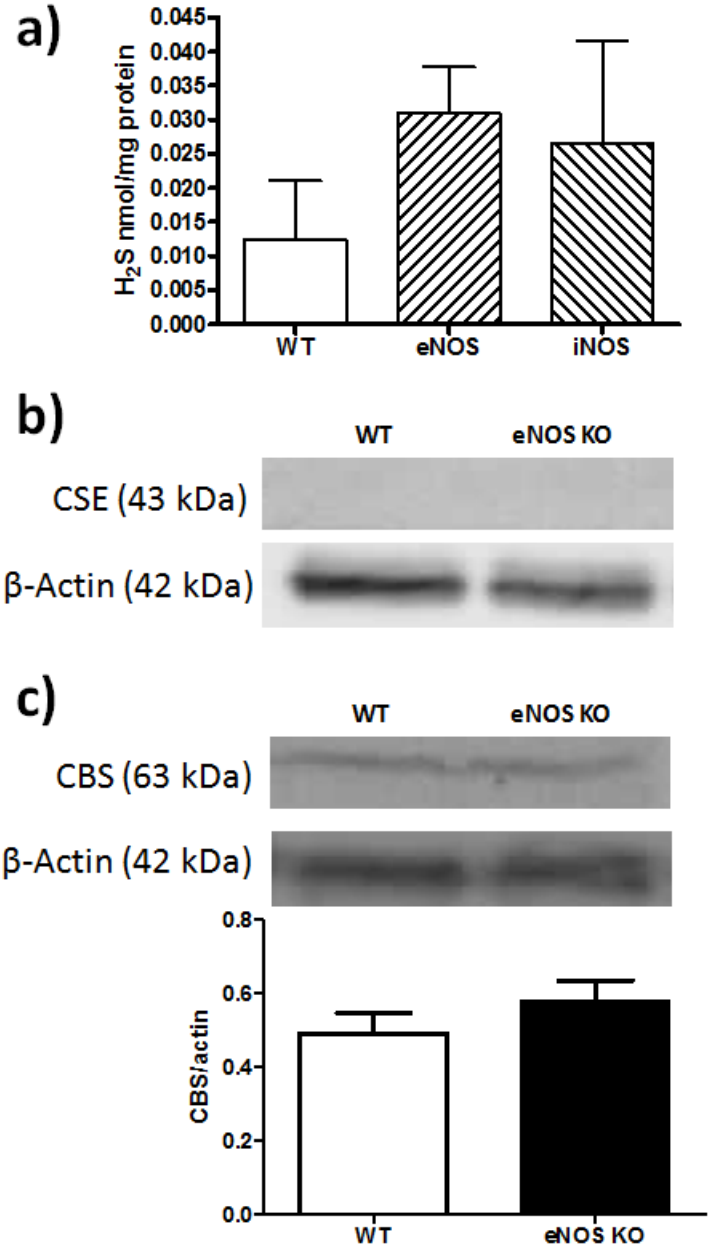

Figure 4.2.7. Graph shows lung a) H_2S enzyme activity (H_2S nmol/mg protein) from WT, $eNOS^{-/-}$ and $iNOS^{-/-}$ mice b) representative Western blot of CSE and β-actin loading control from $eNOS^{-/-}$ and WT lung tissue (40 µg) c) representative Western blot of CBS and β-actin loading control with graphs representing densitometry values of CBS standardised to β-actin from $eNOS^{-/-}$ and WT lung tissue (40 µg). All data are shown as mean ± SEM, n = 6.

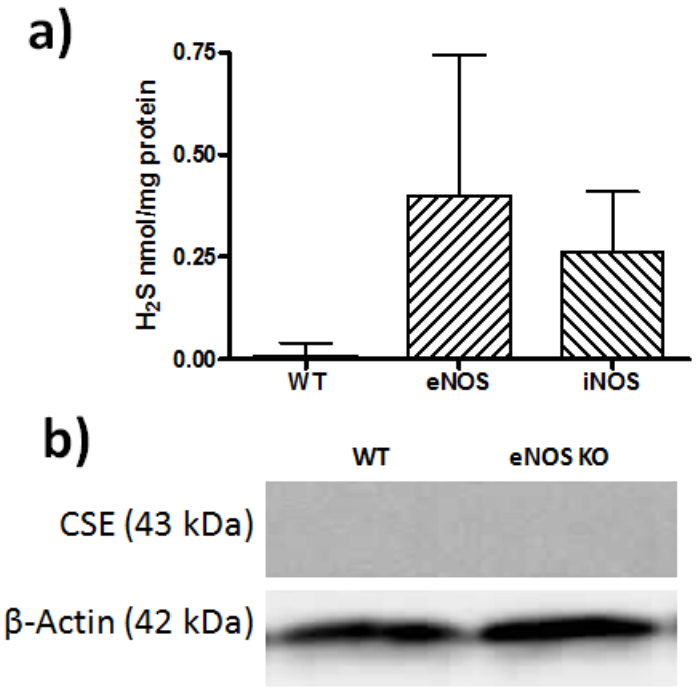

Figure 4.2.8. a) Graph shows H_2S enzyme activity in spleen, measured as H_2S nmol/mg protein, in WT, eNOS$^{-/-}$ KO and iNOS$^{-/-}$ KO mice b) representative Western blot of CSE and β-actin loading control of spleen (50 µg) tissue from WT and eNOS$^{-/-}$ mice. All data are shown as mean ± SEM, n = 6.

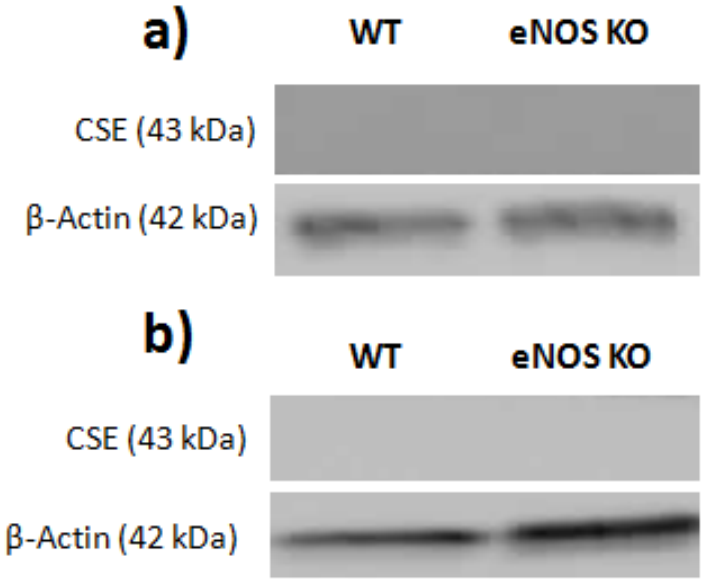

Figure 4.2.9. Representative Western blots from a) heart (50 µg) and b) aorta (20 µg) from WT and eNOS$^{-/-}$ KO mice. Image shows representative blot of from n = 6 mice.

4.3. Discussion

The main findings from this chapter are:

1. H_2S synthesising activity is different between different organs of WT mice
2. There is increased H_2S synthesising activity in $eNOS^{-/-}$ but not $iNOS^{-/-}$ c.f. WT
3. H_2S synthesising activity assay correlates with CSE but not CBS protein expression

The results presented in this chapter indicate that H_2S synthesising activity correlates with CSE but not CBS expression and that this activity/expression is tissue dependent. Liver, kidney, small intestine and stomach were the predominant tissues tested that generated measurable levels of H_2S above the limit of detection. Furthermore, H_2S synthesising activity and CSE expression were up-regulated in $eNOS^{-/-}$ but not $iNOS^{-/-}$ mice. This is consistent with the hypothesis that H_2S synthesis compensates for loss basal $^{\bullet}NO$ signalling. The observation in iNOS KO mice may not be surprising as iNOS is not constitutively expressed and in general is only transcribed during inflammation (discussed later).

4.3.1. H_2S synthesising enzyme activity and CSE/CBS whole body expression profile in WT mice

The organ with the highest H_2S enzyme activity was liver followed by kidney, small intestine, stomach, and brain with negligible and largely variable amounts of H_2S synthesising enzyme activity in the lung and spleen (Figure 4.2.1 a). Similarly, CSE protein expression followed a similar trend to H_2S synthesising enzyme activity, and

was predominately expressed in the liver followed by kidney, and to a lesser extent small intestine and stomach, with undetectable amounts of CSE protein in other tissues. These findings correlate with a previous study which demonstrated a similar CSE protein and H_2S synthesising activity expression profile in both mouse and rat tissues (Ishii, et al. 2004). Interestingly in the latter study, although brain, lung and heart tissue did not express CSE protein, CSE transcripts were detectable (Ishii, et al. 2004).

In contrast to CSE expression, in the current study CBS protein was predominately located in the brain tissue with smaller variable amounts in lung. Others have also described CBS protein and mRNA in mouse hippocampus and purkinje cells of the cerebellum (Robert, et al. 2003b). CBS message has also been described in mouse lung tissue (Kumaraswamy, et al. 2009). CBS mRNA (Bao, et al. 1998, Quere, et al. 1999) and protein (Robert, et al. 2003a, Vitvitsky, et al. 2007) have previously been described in the liver and kidney tissue. However, in the present study CBS protein was undetectable in liver and kidney lysates, although this may be attributable to differences in the epitope recognition sites of the antibodies utilised. Indeed, in most of the studies analysing CBS protein expression, custom made antibodies were developed whilst a commercially developed antibody (Abnova) was used in the present study. Another study utilising a similar strain of mouse (C57BL6J) to the current study (C57BL/6) also described a strong CBS band for brain tissue and a faint protein band for liver tissue, and the absence of a CBS band for kidney tissue (Al-Magableh and Hart 2011). Interestingly, CBS is thought to be 60 and 20-fold lower than CSE in the liver and kidney respectively (Kabil, et al. 2011). These reported differences in relative expression of these two proteins may account for the lack of detectable CBS

protein in these tissues. Indeed, CBS mRNA was detected in human liver as described in Chapter 3.

Irrespective of these slight differences in CBS protein expression presented within this chapter with that of other published findings, the H_2S enzyme activity assay in the current study appeared to correlate with CSE expression but not CBS expression. This suggests that the current assay used to measure H_2S enzyme activity is bias towards measuring the activity of CSE. Indeed it has been suggested that the preferred H_2S generating reaction for CBS may be the condensation of cysteine and homocysteine and the predominant pathway for H_2S generation by CSE is through the α, β-elimination reaction of cysteine (Chiku, et al. 2009, Singh, et al. 2009). In the current study, only L-cysteine was used as a substrate for tissue H_2S generation. Moreover, the activity of CBS may also depend on S-adenosyl methionine (SAM) in addition to P5'P (Chen, et al. 2004). Therefore an alternative assay utilising both homocysteine and cysteine in the presence of SAM and P5'P may better reflect the activity of both CSE and CBS enzymes.

4.3.2. H_2S synthesising enzyme activity in $iNOS^{-/-}$ mice

In the current study, H_2S synthesising enzyme activity was not altered in $iNOS^{-/-}$ mice compared to WT in most tissues: brain, lung, liver, spleen, stomach and small intestine. iNOS is not constitutively expressed in most tissues and given that the hypothesis is that H_2S generation will compensate for the loss of $^{\bullet}NO$ production, it is not surprising that there was no change in H_2S synthesising activity in these animals. Whether a difference in H_2S enzyme activity in $iNOS^{-/-}$ mice would transpire in response to a noxious stimulus, such as LPS, is another area that requires further investigation.

Interestingly, a significant reduction in H_2S enzyme activity was noted in kidney tissue of $iNOS^{-/-}$ compared to WT. The reason for this reduction in H_2S enzyme activity is unknown. However, there have been reports that iNOS is constitutively expressed in kidney (Vaziri, et al. 1998, Poljakovic, et al. 2001, Chou, et al. 2002), which may suggest an alternative role for iNOS in the kidney other than in immunological defence. Indeed, further studies are required to examine the iNOS organ expression profile in WT mice. Interestingly, CSE and CBS expression, like iNOS, is found primarily in the renal tubules (Ishii, et al. 2004). $iNOS^{-/-}$ KO mice have been demonstrated to have a reduced fluid absorption, demonstrated by a reduction in Na^+ absorption (Wang 2002b). Conversely, H_2S has also been shown to inhibit Na^+ absorption (Xia, et al. 2009). It can be speculated that the reduction in H_2S enzyme activity in the $iNOS^{-/-}$ KO mice, may be a compensatory mechanism to reduce further loss of ions such as Na^+ in the urine.

4.3.3. H_2S enzyme activity and CSE expression in $eNOS^{-/-}$ mice CSE

In contrast to $iNOS^{-/-}$ mice, significant differences in H_2S synthesising activity and CSE expression was described in a number of tissues derived from $eNOS^{-/-}$ mice compared to WT control tissue. These differences will be discussed in an organ specific manner.

a) Liver

The liver has many functions such as the biosynthesis of proteins and the removal of toxic substances. As a result this organ is a large source of enzymes involved in amino acid synthesis and metabolism. Indeed, both CSE and CBS enzymes are intimately involved in the transulfuration pathway of converting the toxic L- homocysteine to L-cysteine (Persa, et al. 2004). The transulfuration pathway is also connected to the

transmethylation pathway between L-methionine and L-homocysteine (Riedijk, et al. 2007). CSE and CBS are therefore vital for the conversion of L-methionine to L-cysteine and for the removal of L-homocysteine. Moreover, CSE may be essential for an adequate supply of cysteine necessary for the synthesis of GSH in the liver (Rao, et al. 1990). It is therefore not surprising why hepatocytes are a large source of these enzymes, and likely to be a reason why liver is one of the largest source of H_2S in the body.

Whether H_2S has other functions in the liver other than being a by-product of cysteine metabolism is probable due to the vast number of biological actions described within the cardiovascular system. Indeed, the liver is a large source of blood vessels. CSE has been described to be present in the hepatocytes as well as the stellate cells of liver, but interestingly not in the sinusoidal endothelial cells of the liver vasculature (Fiorucci, et al. 2005b). Sinusoidal resistance is regulated by regulating the contraction and relaxation of hepatic stellate cells surrounding the sinusoidal endothelial cells (Fiorucci, et al. 2005b). CSE protein expression has been shown to be down regulated in hepatic stellate cells of rats with liver portal hypertension, suggesting that H_2S derived from CSE plays a role in the maintenance of perfusion pressure in the liver vasculature (Fiorucci, et al. 2005b).

In the current study, an up-regulation of CSE protein expression and H_2S enzyme activity was described in the liver tissue of $eNOS^{-/-}$ KO mice compared to WT (Figure 4.2.2). eNOS is located in the endothelial cells of the vasculature of the liver as well as hepatocytes (McNaughton, et al. 2002), and the predominant role for eNOS in the liver is thought to be the regulation of hepatic perfusion (Li and Billiar 1999, Shah, et al. 2004). Indeed, $^{\bullet}NO$ inhibitor N omega-nitro-L-arginine (NNA) has been

demonstrated to increase ($P<0.05$) baseline portal pressure compared to control (Mittal, et al. 1994). Moreover, other studies have suggested an up-regulation of eNOS activity in rats induced with portal vein ligation for two weeks (Cahill, et al. 1996).

The liver is a large vascular organ and both $^{\bullet}NO$ and H_2S are vasodilators and both have been suggested to regulate hepatic perfusion. The up-regulation of H_2S enzyme activity and CSE protein expression in the current study may suggest a compensatory mechanism to normalise hepatic perfusion pressure for the loss of $^{\bullet}NO$ production in the $eNOS^{-/-}$ KO mice. However, further study is required to confirm these conclusions. Whether an up-regulation of CSE occurs in the hepatocytes, hepatic stellate cells or both, requires further study.

b) Kidney

The core functions of the kidney are to remove filter, reabsorb and secrete fluids and free amino acids of the body. Another important function of the kidney is for the metabolism and removal of amino acids, such as homocysteine (Urquhart and House 2007). In the rat cortical tubules, homocysteine is metabolised predominately by the transulfuration pathway (House, et al. 1997). However, whether the kidney is a major organ for homocysteine metabolism in humans is debatable (Urquhart and House 2007).

Interestingly, in rats CBS is localised largely in the outer cortex and predominately in the proximal convoluted tubule segment of the kidney (House, et al. 1997). In contrast, CSE is located largely in the outer medulla and primarily in the proximal straight tubules of the nephron (House, et al. 1997). This may suggest CSE and CBS are involved in regulating tubular function in the kidney. In the adult rodent

eNOS is located exclusively in the endothelial cell lining of all blood vessels, including glomerular capillaries and peritubular capillaries of the medulla (Han, et al. 2005a, Forbes, et al. 2007), suggesting a role for eNOS in the regulation of blood flow in the kidney.

Interestingly it has been demonstrated that infusing AOAA (CBS inhibitor) and PAG (CSE inhibitor) together, both 0.5 μmol/min/kg, significantly reduced glomerular filtration rate, urinary sodium and potassium excretion (Xia, et al. 2009). Moreover, NaHS was shown to directly inhibit $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+ ATPase activity (Xia, et al. 2009), suggesting that H_2S can inhibit the reabsorption of ions such as Na^+. These authors suggested that H_2S may have roles in both vascular and tubular actions in the kidney (Xia, et al. 2009). However, in the current study, no change in H_2S enzyme activity or CSE protein expression was noted in $eNOS^{-/-}$ compared to WT mice. This may suggest that although, H_2S produced by CSE in the kidney may contribute to the vascular regulation of the kidney, the role for H_2S in vascular regulation may be minimal. However, other compensatory mechanisms may replace the role for eNOS in the kidney. Indeed, as described before (Discussion 4.3.2) iNOS is constitutively active in the kidney (Vaziri, et al. 1998, Poljakovic, et al. 2001, Chou, et al. 2002) and it can be hypothesised that iNOS may directly compensate for the loss of $^{\bullet}NO$ in the $eNOS^{-/-}$. However, further study is required to confirm this.

Overall, in the current study, there was no significant difference in H_2S enzyme activity or CSE expression in the kidney of $eNOS^{-/-}$ KO mice compared to WT. The reason behind this is unknown. However it is possible that the regulation of renal vascular function may not be the primary role for CSE/CBS in the kidney.

c) Gastrointestinal tract

The predominant role of the GIT is for the digestion of food, absorption of nutrients, and the extrusion of waste. In the rat colon CBS is found predominately in the fibroblast and smooth muscle cells (Wallace, et al. 2009). Whereas CSE expression is more diffuse and was shown to be located in blood vessels, epithelial, crypt and goblet cells (Wallace, et al. 2009).

In the current study, a notable amount of H_2S synthesising enzyme activity was described in the stomach and small intestine of WT mice (Figure 4.2.1). Bacteria are a potential source of H_2S enzyme activity in the gastrointestinal tract as they themselves are capable of synthesising H_2S (Gibson, et al. 1990, Pitcher and Cummings 1996). The stomach and small intestine was thoroughly cleaned of their contents, leaving predominately the bulk tissue mass, therefore, bacteria is unlikely to be the main source of H_2S synthesising enzyme activity in the current study. Moreover, although bacteria reside throughout the GIT, acid and bile is thought to hinder bacterial colonisation in the stomach and proximal small intestine (O'Hara and Shanahan 2006). Instead, bacteria predominately colonise in the distal end of the small intestine and large intestine (O'Hara and Shanahan 2006), and is a reason why the large intestine and proximal end of the small intestine was not unitised in the current study.

$^{\bullet}NO$ derived from eNOS has been implicated in maintaining the mucosal barrier of the GIT (Shah, et al. 2004). NG-nitro-L-arginine methyl ester (L-NAME) has been shown to increase basal epithelial permeability in the small intestine of the cat (Kubes 1992) and in the rat (Kanwar, et al. 1994). Moreover, the effects of L-NAME on epithelial permeability can be reversed on incubation with $^{\bullet}NO$ donor, sodium

nitrossprusside (SNP) (Kubes 1992). Which may suggest that basal constitutive $^{\bullet}$NO production, is involved in protection of the mucosal barrier function.

Interestingly, eNOS$^{-/-}$ KO mice are less susceptible to dextran sodium sulfate-induced colitis than iNOS$^{-/-}$ or nNOS$^{-/-}$ KO mice, which may suggest $^{\bullet}$NO derived from eNOS may promote colitis (Beck, et al. 2004). However, in the current study, an increase in H_2S synthesising enzyme activity was noted in stomach and small intestine of the eNOS$^{-/-}$ compared to WT. Exogenous and endogenous H_2S has previously been demonstrated to reduce the severity of colitis or gastric inflammation in a number of models (Fiorucci, et al. 2005a, Wallace, et al. 2007b, Medeiros, et al. 2009, Wallace, et al. 2009), and may be the reason for the resistance against colitis in the eNOS$^{-/-}$ KO mice (Beck, et al. 2004).

It is possible the compensatory increase in H_2S synthesising enzyme activity in the GIT of eNOS$^{-/-}$ KO mice described in the current study, is a compensatory protective mechanism for maintenance of the GIT barrier. Furthermore, this compensatory up-regulation of H_2S in the GIT of eNOS$^{-/-}$ KO mice may be the reason for the protection against experimental colitis in eNOS$^{-/-}$ KO mice demonstrated in previous studies (Beck, et al. 2004). However, studies measuring H_2S synthesising enzyme activity in eNOS$^{-/-}$ with gastric damage are required to confirm this hypothesis.

d) Spleen, brain, lung, heart and aorta

In the current study no significant difference in the H_2S synthesising enzyme activity between eNOS$^{-/-}$ KO mice and WT was noted in the spleen, brain or lung tissue (Figure 4.2.6 – 4.2.8). It should be noted that the H_2S enzyme assay was not carried out in: heart due accidental loss of tissue; thoracic aorta as the tissue mass was too small to

carry out the H_2S enzyme assay. H_2S produced from these tissues were at the limit of detection, with ~5 µM H_2S detected in brain tissue and ≤ 2 µM H_2S detected from spleen and lung tissue (all tissues 15% w/v). Indeed, the small amounts of H_2S produced in these tissues were small and variable making it difficult to determine a difference between WT and $eNOS^{-/-}$ KO mice if there was a difference. Similar to the current study, others have also described limited H_2S enzyme activity in the spleen, brain and lung, and no H_2S enzyme activity in heart tissue (Ishii, et al. 2004).

CSE protein expression was not detected in heart, aorta, spleen, brain and lung tissue in the current study (Figure 4.2.6 – 4.2.9). This correlated with another study that demonstrated the absence of CSE protein in the brain, heart, spleen and lung tissue (Ishii, et al. 2004). Although CSE protein was not detected, in the same study, CSE mRNA was present in the brain, heart and lung tissue (Ishii, et al. 2004). While CSE protein was not detected in mouse aorta in the current study, CSE protein expression has previously been demonstrated in rat aorta by western blotting (Cheng, et al. 2004), and in mouse aorta by immunohistochemistry (Yang, et al. 2008).

The reason for the differential location and density of CSE in peripheral tissue is unknown. It is possible the role for CSE in the brain, heart, spleen and lung may be of lesser importance than in the liver, kidney and GIT where CSE is expression is detectable. However, it is possible that the sensitivity of the detection methods for CSE protein and H_2S generation is a reason why CSE/H_2S was not detected in the brain, heart, spleen and lung tissue.

CBS protein was only detectable in the brain and lung tissue. In both brain and lung tissue, there was no significant difference in CBS protein expression between WT

and eNOS$^{-/-}$ KO mice (Figure 4.2.6 and 4.2.7). This is consistent for a predominant role for CBS in the central nervous system rather than the cardiovascular system.

4.3.4. Summary and conclusion

In summary, an up-regulation of H_2S synthesising enzyme activity was noted in tissues capable of generating robust and measureable concentrations of H_2S (10 – 190 µM), that is the liver, stomach and small intestine; the exception being kidney, where no change in H_2S synthesising enzyme activity or CSE expression was observed. The data from this descriptive study suggests that signalling mediated by $^{\bullet}NO$ can be compensated by H_2S. However, further functional studies are required to confirm these findings. The examination of the vascular reactivity of vessels derived from eNOS$^{-/-}$ on exogenous H_2S administration would further provide further evidence for a functional compensatory effect of H_2S for eNOS. Furthermore, the global examination of eNOS protein expression and total NOS activity would provide insight as to whether NOS has a similar expression profile to H_2S synthesising activity. Finally, one should keep in mind that the H_2S synthesising enzyme assay used in the current study is likely to reflect H_2S generation by CSE (rather than both CSE and CBS) and therefore is not a true representation of the total H_2S synthesising capacity of tissues and is a limitation in the current study.

CHAPTER 5: Effect of H_2S Donors On Adhesion Molecule Expression

5. Introduction

The role of H_2S in inflammation is unclear. H_2S donors have shown to exert both pro-inflammatory (Bhatia, et al. 2006, Tamizhselvi, et al. 2007, Zhang, et al. 2007b, Zhi, et al. 2007, Zhang, et al. 2008) and anti-inflammatory action (Fiorucci, et al. 2005a, Zanardo, et al. 2006, Wallace, et al. 2007b, Andruski, et al. 2008, Chen, et al. 2009, Wallace, et al. 2009, Kloesch, et al. 2010). Similarly, H_2S inhibitors have also demonstrated pro-inflammatory (Fiorucci, et al. 2005a, Zanardo, et al. 2006, Wallace, et al. 2009) and anti-inflammatory action (Bhatia, et al. 2005, Collin, et al. 2005, Li, et al. 2005, Bhatia, et al. 2006, Tamizhselvi, et al. 2007, Zhang, et al. 2007b, Mok and Moore 2008, Zhang, et al. 2008).

Both inhibitors of H_2S synthesising enzymes and H_2S donors have their flaws. Most of the antagonists used to inhibit CSE or CBS, inhibit the pyridoxal 5' phosphate binding site which may result in non-selective inhibition of other pyridoxal 5' phosphate dependent enzymes (Tanase and Morino 1976, Rej 1977, Burnett, et al. 1980, Loscher 1981, Hamel, et al. 1982).

NaHS is the most commonly used H_2S donor used in deciphering the actions of H_2S in biological systems. One limitation with the use of NaHS is that cells may be subject to instantaneously high concentrations of H_2S, which can be cytotoxic (Beauchamp, et al. 1984, Eghbal, et al. 2004, Cao, et al. 2006, Adhikari and Bhatia 2008). Moreover, the volatile properties of H_2S can make handling difficult, potentially leading to inconsistencies between individual investigators. Indeed, within several minutes of solubilising NaHS, the concentration of H_2S released from NaHS would have been dissipated into the atmosphere leaving much less H_2S present in solution (see

results later). Moreover, the instantaneous release of H_2S from NaHS does not mimic the release of endogenous H_2S.

A novel organic slow releasing H_2S donor GYY4137 has recently been characterised (Li, et al. 2008b). GYY4137 releases H_2S at a rate of 4 nmol/25 min H_2S at 37^{o}C pH 7.4 (Li, et al. 2008b). In addition, GYY4137 (100 µM) did not significantly affect rat vascular smooth muscle cell viability in culture (Li, et al. 2008b). Furthermore, treating spontaneously hypertensive rats with a daily dose of GYY4137 (133 µmol/kg, i.p.) over 14 days did not obviously induce abnormal behavioural changes (Li, et al. 2008b). The slower progressive release of H_2S from GYY4137 may provide further insight into the role of H_2S in inflammation. Indeed, in a later study, GYY4137 was shown to have an anti-inflammatory effect in an *in vivo* model of endotoxin shock in the rat (Li, et al. 2009b). The current study will examine the effects of GYY4137 on *in vitro* and *in vivo* models of inflammation.

Hypothesis: GYY4137 will exert anti-inflammatory effects in both *in vitro* and *in vivo* models of inflammation. Aim:

1. Determine the effects of GYY4137 and NaHS on adhesion molecule expression from HUVEC and further characterise the mechanisms behind the actions of GYY4137
2. Explore the effects of GYY4137 in the carrageenan hindpaw model of inflammation in the mouse, specifically: oedema, mechanical hyperalgesia and myeloperoxidase (MPO) activity.

5.1. Materials and Methods

5.1.1. GYY4137

GYY4137 (morpholin-4-ium-4-methoxyphenyl(morpholino) phosphinodithioate was synthesised by Dr. Choon-Hong Tan (National University of Singapore, Singapore) on the basis of the structure of Lawesson's compound as described by Li, et al. 2008b (Figure 5.1.1). GYY4137 was first used as an accelerant for the vulcanisation of rubber (Li, et al. 2008b). GYY4137 was identified from a screen of compounds in 2008, as a water soluble slow releasing H_2S donor (Li, et al. 2008b). Until 2008, GYY4137 had not been described to have any biological effects (Li, et al. 2008b).

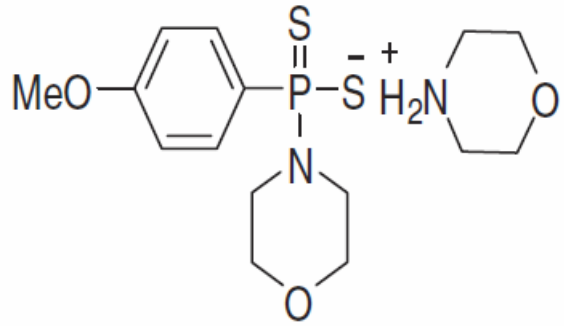

Figure 5.1.1. Structure of GYY4137. Taken from (Li, et al. 2008b)

5.1.2. Animals

Female CD1 mice (Charles river, 25g) were housed in King's College London Biological Services Unit (Waterloo) and kept in a climatically controlled environment (45% humidity, 20^{o}C, 12 h light cycle). Food and water was provided *ad libitum*. Aged matched female mice were used for all studies. All experiments were carried out according to the 1986 UK Home Office Animals Scientific Procedures Act.

5.1.3. Carrageenan hindpaw model

The effects of GYY4137 were assessed using the mouse model of carrageenan induced paw inflammation (Campo, et al. 2009). Following induction of anaesthesia (isoflurane 4% v/v), the hindpaw of mice were injected with 50 µl carrageenan (1% w/v in saline, 29 G needle) and the contra-lateral paw with 50 µl saline control (0.9% w/v sodium chloride) (Dufton, et al. 2010). Mice were left undisturbed and allowed to recover for 1 h. GYY4137 (50 mg/kg, 10 ml/kg, i.p.) (Li, et al. 2008b) was administered into conscious animal's 1 h post- intraplantar (i.pl.) injection. All i.p. and i.pl injections were blinded and randomised.

The extent of inflammation was quantified using Calipers (Mitutoyo, Japan) and paw and ankle thickness were determined at 0.5, 1.5, 3.5 and 5.5 h after carrageenan injection. To examine whether GYY4137 had an effect on mechanical hyperalgesia, in the same animals, a Von Frey-type filament was used to measure mechanical hyperalgesia at 1, 2, 4 and 6 h after saline or carrageenan i.pl injection (see Method 5.1.4 for further detail). Mice were subsequently killed by cervical dislocation under anaesthesia (isoflurane 4% v/v). Paws were subsequently cut at the ankles, weighed, snap frozen in liquid nitrogen and stored at -80^{o}C for biochemical analysis (Methods 5.1.5 and 5.1.7).

5.1.4. Mechanical hyperalgesia

Conscious mice were allowed to acclimate for 30 min in individual chambers (L x W x H, 10 x 9 x 16 cm) containing metal mesh flooring. A dynamic plantar aesthesiometer (Ugo Basile, Milan, Italy) was used to determine the nociceptive threshold to a mechanical stimulus via a Von Frey-type filament (0.5 mm diameter) attached to an

actuator. The Von Frey-type filament was applied at the plantar surface until the mouse removed its hindpaw and the force at which the hindpaw was withdrawn (g) was subsequently recorded. An average of 4 withdrawal readings per hindlimb was recorded as the response to a mechanical stimulus. The device was calibrated to apply an increasing rate of 5 g/sec and a maximum force 50 g (cut off pressure to avoid tissue damage).

5.1.5. MPO assay in mouse hindpaws

Myeloperoxidase (MPO) was used as an indicator of leukocyte accumulation in the mouse hindpaw. 3,3',5,5'-tetramethylbenzidine (TMB) is a substrate used to determine peroxidase activity (Andrews and Krinsky 1982). The activity of MPO was assayed using the H_2O_2-oxidation of TMB. In the presence of MPO, H_2O_2 is hydrolysed to form oxygen radicals that directly oxidises TMB. When TMB is oxidised it emits a blue colour which is most stable ≤ pH 6.2 (Andrews and Krinsky 1982).

Hindpaws of the mice were homogenised (200 mg/ml) in a homogenising buffer (NaCl 600 mM, KH_2PO_4 600 mM, Na_2HPO_4 66 mM, hexadecyltrimethyl-ammonium bromide (HTAB) 0.5% v/v) using a polytron homogeniser (Brinkman). The samples were then centrifuged at 13,000 g for 10 min at 4^{o}C and the supernatant was diluted 1:50 in saline. Diluted supernatant or purified MPO standard (25 µl) was incubated with MPO buffer (25 µl, Na_2HPO_4 6.6 mM, KH_2PO_4 60.1 mM, HTAB 0.5% v/v, pH 6) and TMB/H_2O_2 100 µl in a 96-well plate. The plate was incubated at room temperature for 30 min and the absorbance was determined at 652 nm. Values were standardised to protein using the BCA protein assay (Method 2.6) to determine units

MPO/mg protein. A standard curve for MPO was constructed from purified MPO derived from human leukocytes (0.1 – 1 units MPO/ml, Sigma M6908).

5.1.6. *In vitro* assay to determine the effects of GYY4137 on purified MPO activity

Purified human MPO (0.75 units MPO/ml in $KHPO_4$ 100 mM, pH 7.4) was incubated with GYY4137 (5 – 500 µM) for 1 h at 37^{o}C. 50 µl of MPO/GYY4137 was incubated with TMB/H_2O_2 substrate for 30 min. Absorbance was read at 652 nm using a spectrophotometer (Tecan).

5.1.7. H_2S synthesising enzyme activity in carrageenan hindpaws

Mouse hindpaws were homogenised in cold potassium phosphate buffer (10% w/v, $KHPO_4$ 100 mM, pH 7.4) and the H_2S synthesising assay was carried out as in the standard protocol (Method 2.11). A Bradford protein assay (Method 2.5) was carried out to determine the H_2S synthesised nmol/mg protein.

5.1.8. Soluble adhesion molecule expression

HUVEC were seeded on gelatine coated 6-well plates (4 x 10^4 cells per well). When confluent, the cells were pre-treated with GYY4137 (31.125 – 500 µM) or NaHS (62.5 – 500 µM) in M199-1% (1 ml) for 1 h prior to IL-1β (10 ng/ml) or LPS (Escherichia coli, serotype O127:B8, 100 ng/ml) stimulation for 24 h. The cell culture medium was subsequently stored and frozen at -80^{o}C. Quantikine ELISA kit (R&D systems) were used to measure human soluble VCAM-1 (DVC00), ICAM-1 (DCD540) and E-selectin (DSLE00). The cell culture medium was assayed without dilution for soluble VCAM-1, ICAM-1 and E-selectin according to manufacturer's instructions.

5.1.9. Surface adhesion molecule expression on HUVEC

HUVEC were seeded in gelatin coated 96-well plates (1 x 10^4 cells/well). Edge wells were not used. When confluent (2-3 days), HUVEC were pre-treated with GYY4137 (31.25 – 5000 μM) or NaHS (62.5 – 5000 μM) for 1 h in M199-1% prior to stimulation with IL-1β (10 ng/ml) for 24 h. The cells were rinsed with PBS once and fixed in 2% v/v formalin (PBS, 100 μl/well) for 20 min at room temperature. The fixed cells were subsequently blocked with 5% w/v albumin (in PBST, PBS with 1% v/v Tween-20) for 1 h. The cells were subsequently incubated with primary antibodies against VCAM-1 (1:500, 5% w/v albumin PBST) or ICAM-1 (1:500, 5% w/v albumin PBST) for 1 h at room temperature. The antibody was aspirated and the plates washed with PBST (1x 1 min, 2x 5 min, 1x 10 min) and 5% w/v albumin (1x 20 min). The wells were subsequently incubated with secondary-HRP linked anti-goat antibody (1:5000, 5% w/v albumin PBST) for 1 h at room temperature. The plate was then washed in PBST (1x 1 min, 2x 5 min, 1x 10 min, 1x 20 min). Equal volumes of TMB (3,3',5,5'-tetramethylbenzidine) and H_2O_2 were mixed (Thermo Scientific). 200 μl of TMB/H_2O_2 substrate was added to each well. At 30 min, the oxidation of TMB by peroxidase was subsequently quantitated spectrophotometrically at 652 nm as an indicator of surface adhesion molecule expression. The TMB was subsequently aspirated and the plate rinsed with water. On the same plate, the crystal violet viability assay was carried out to determine cell density (Method 5.1.11). Graphs of adhesion molecule expression were subsequently plotted as absorbance measured at 652 nm (TMB) over absorbance at 610 nm (crystal violet).

5.1.10. Crystal violet viability assay

Crystal violet staining is used as an indicator of cell number. HUVEC (1 x 10^4 cells/well) were fixed in formalin (2% v/v in PBS, 100 µl/well) for 20 min and subsequently stained with 100 µl crystal violet (0.2% w/v crystal violet in 2% v/v aqueous ethanol) for 10 min at room temperature. The crystal violet solution was aspirated and the plate was rinsed in water to remove excess stain. Subsequently, 200 µl of 0.5% w/v SDS in 50% v/v ethanol was added to each well and incubated at 37^{o}C for 1 h to dissolve the crystal violet stained cell membranes. The absorbance was quantified using a spectrophotometer at 610 nm.

5.1.11. ATP viability assay

To ensure HUVEC were still metabolically active in the presence of H_2S donors, ATP production was used as an endpoint marker for cell viability. HUVEC were seeded in opaque-walled, clear bottom 96-well plates (1 x 10^4 cells/well). When confluent, the cells were treated with GYY4137 or NaHS (Method 5.1.8). The CellTiter-Glo Luminescent Cell Viability assay (Promega) was used to determine ATP production in cells. Lyophilized CellTiter-Glo Substrate, containing recombinant luciferase and luciferin substrate, was reconstituted in CellTiter-Glo buffer to form the working reagent. In the presence of ATP, luciferase catalyses the conversion of:

$$\text{Luciferin} + \text{ATP} + O_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{light}$$

The luminescent signal is then subsequently quantitated by a luminometer. The protocol was carried out according to manufacturer's instructions. In brief, the plate containing treated HUVEC was left to equilibrate at room temperature for 30 min. 100

µl of working reagent (reconstituted luciferase/luciferin) was added directly to 100 µl of cell culture medium, and left on the shaker for 10 min before relative light units/second was measured using a microplate luminometer (Hidex).

5.1.12. IKB-α degradation in HUVEC

HUVEC were pre-treated with GYY4137 (125 or 500 µM) or vehicle (M199-1%) 1 h prior to stimulation with IL-1β (10 ng/ml) for 15, 30, 60 or 120 min. Cells were subsequently rinsed twice with PBS and lysed in lysis buffer. Western blots for IKB-α (43 kDa) were carried out.

5.1.13. COX-2 expression in HUVEC

HUVEC were pre-treated with GYY4137 (125 – 500 µM) or vehicle (M199-1%) 1 h prior to stimulation with IL-1β (10 ng/ml) for 24 h. Cells were rinsed twice with PBS and lysed in lysis buffer. Western blots for COX-2 protein expression (72 kDa) were subsequently carried out.

5.1.14. Griess assay for nitrate/nitrite from RAW264.7 cells

RAW264.7 cells were plated on 6-well plates and treated with 1 µg/ml LPS (Escherichia coli 055:B5) in SF-DMEM respectively for 24 h (Whiteman, et al. 2010c). Cell culture medium was assessed for nitrite/nitrate. Cell culture medium 100 µl, was incubated with Griess reagent 100 µl (1:1 ratio of 2% w/v sulfanilamide in 5%v/v H_3PO_4: 0.2% w/v naphthylethylenediamine dihydrochloride in 5%v/v H_3PO_4) in a 96-well plate in the dark at room temperature for 15 min. A standard curve for nitrite was constructed using sodium nitrite (4 – 500 µM). Absorbance was quantified using a spectrophotometer at 540 nm.

5.2. Results

5.2.1. Comparing H_2S release from GYY4137 and NaHS

The rate of H_2S release of from GYY4137 and NaHS was first compared. GYY4137 (31.25 – 5000 µM) and NaHS (500 µM) were dissolved in cell culture medium (M199-1%) and incubated at 37^{o}C. The release of H_2S from GYY4137 and NaHS was subsequently determined by the methylene blue assay. On exposing NaHS to solution, the concentration of H_2S measured followed an exponential decay. Within 10 min approximately 50% of the H_2S released from NaHS remained in solution (198.10 ± 5.89 µM H_2S, n = 3) and after 1 h the H_2S was undetectable (Figure 5.2.1 b). In contrast to NaHS, H_2S released from GYY4137 was sustained for 24 h (Figure 5.2.1 a).

5.2.2. Effect of H_2S on E-selectin, VCAM-1 and ICAM-1

To determine the effects of H_2S donors on adhesion molecule expression, HUVEC were pre-treated with GYY4137 or NaHS for 1 h prior to stimulation with IL-1β (10 ng/ml) for 24 h. ELISAs for E-selectin, VCAM-1 and ICAM-1 were subsequently carried out. Soluble adhesion molecules were measured in the cell culture medium, whereas surface adhesion molecules were measured from the cell surface of HUVEC.

a) H_2S donors on E-selectin expression

IL-1β caused a significant ($P<0.05$) rise in soluble E-selectin (0.00 c.f. 2.49 ± 0.53 ng/ml, n = 4, Figure 5.2.2). Pre-treating HUVEC with GYY4137 or NaHS (up to 500 µM) did not significantly alter soluble E-selectin accumulation compared to control (Figure 5.2.2). No further work was carried out with HUVEC on E-selectin expression.

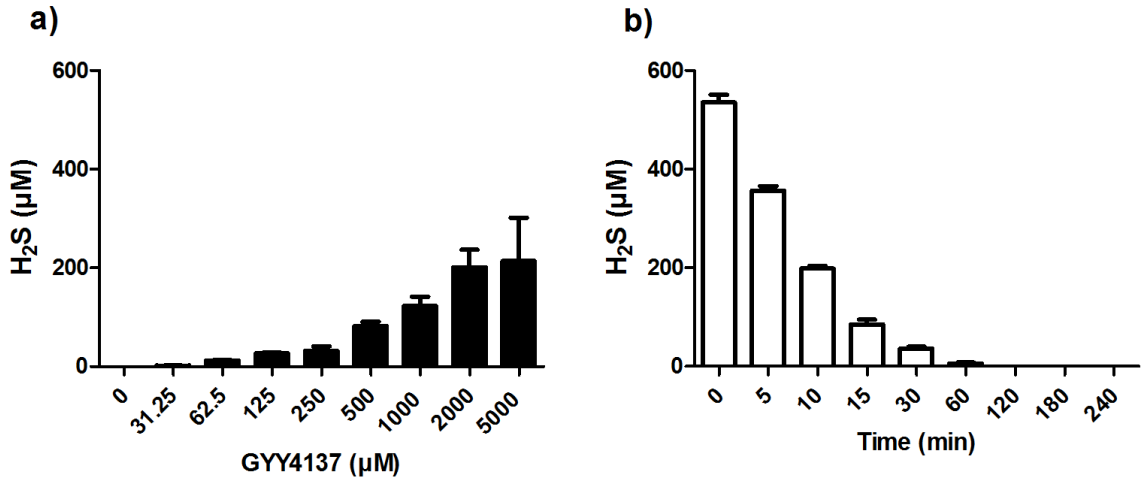

Figure 5.2.1. GYY4137 and NaHS were dissolved in cell culture medium (M199-1%) and incubated at 37^{o}C. The methylene blue assay was used to detect H_2S at various time points a) H_2S released from GYY4137 (31.25 – 5000 µM) is detectable after 24 h b) H_2S released from NaHS (500 µM) is no longer present after 60 min. Values show mean ± SEM, n = 3.

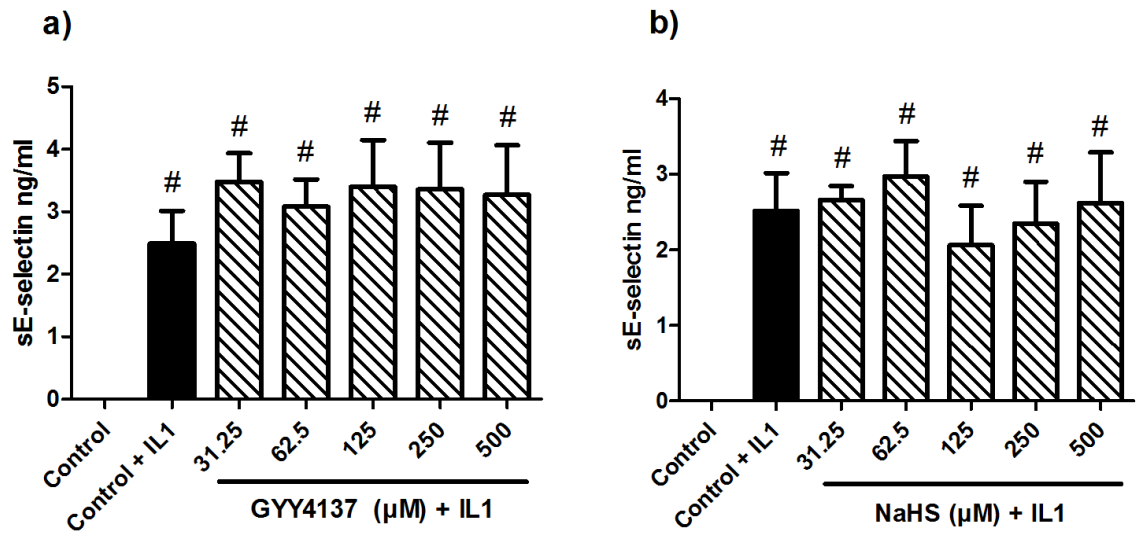

Figure 5.2.2. HUVEC were pre-treated with GYY4137 (31.25 – 500 µM) or NaHS (31.25 – 500 µM) for 1 h prior to stimulation with IL-1β (10 ng/ml) for 24 h. Cell culture medium was assayed for soluble E-Selectin using a commercial ELISA. Results show soluble E-selectin ng/ml as mean ± SEM, n = 4, #P<0.05 c.f. control.

b) H_2S donors on ICAM-1 expression

IL-1β caused a significant (P<0.05) rise in soluble ICAM-1 (1.30 ± 0.50 c.f. 4.43 ± 0.86 ng/ml, n = 4, Figure 5.2.3) compared to control. However, neither GYY4137 nor NaHS (32.25 – 500 μM) pre-treatment significantly reduced IL-1β induced soluble ICAM-1 expression (Figure 5.2.3).

GYY4137 appeared to trend towards a reduction in soluble ICAM-1 expression. Therefore further work was undertaken to characterise the effects of H_2S donors on IL-1β induced surface ICAM-1 expression on HUVEC. Surface ICAM-1 was maximally expressed at 24 h (Figure 5.2.5), as shown by a 1.15 ± 0.03 fold increase compared to control cells. Although GYY4137 and NaHS (500 μM) did not statistically inhibit soluble ICAM-1 expression (Figure 5.2.3), GYY4137 (5000 μM % inhibition 98.91 ± 20.88, P<0.05, n = 8, Figure 5.2.6 c) and NaHS (2000 μM, % inhibition 72.85 ± 14.32, P<0.05, n = 8, Figure 5.2.6 d) significantly inhibited surface ICAM-1 expression.

c) H_2S donors on VCAM-1 expression

IL-1β caused a significant rise in soluble VCAM-1 expression (14.79 ± 1.34 c.f. 34.69 ± 5.69 ng/ml, P<0.05, n = 4, Figure 5.2.4). This rise in soluble VCAM-1 expression induced by IL-1β was significantly inhibited by GYY4137 pre-treatment (62.5 μM, % inhibition 70.37 ± 13.22, P<0.05, n = 4, Figure 5.2.4 a). In contrast to GYY4137, NaHS (500 μM) did not significantly reduce soluble VCAM-1 (Figure 5.2.4 b).

To determine whether GYY4137 or NaHS (32.25 – 500 μM) effects LPS induced soluble VCAM-1 expression, HUVEC were pre-treated with GYY4137 or NaHS (32.5 – 500 μM) and stimulated with LPS (100 ng/ml) for 24 h. LPS significantly increased soluble VCAM-1 expression (10.25 ± 0.74 c.f. 16.61 ± 1.59 ng/ml, P<0.05, n = 4, Figure 5.2.4 c). Pre-treatment with GYY4137 significantly reduced soluble VCAM-1 induced by

LPS in HUVEC (125 μM, % inhibition 101.75 ± 19.27, $P<0.05$, n = 4, Figure 5.2.4 c). In contrast, NaHS did not significantly reduce LPS-induced soluble VCAM-1 expression (Figure 5.2.4 d).

Further work was undertaken to characterise the effects of H_2S donors on surface VCAM-1 expression on HUVEC. Surface VCAM-1 was maximally expressed at 24 h (Figure 5.2.5), as shown by a 1.22 ± 0.07 fold increase compared to unstimulated cells. Similar to the soluble VCAM-1 data, GYY4137 significantly reduced surface VCAM-1 expression (500 μM, % inhibition 52.05 ± 6.59, $P<0.05$, n = 8, Figure 5.2.6 a). Interestingly, higher concentrations of GYY4137 were necessary to significantly reduce surface VCAM-1 expression (500 μM, Figure 5.2.6 a) compared to soluble VCAM-1 (62.5 μM, Figure 5.2.4 a).

Although NaHS (500 μM) did not significantly affect soluble VCAM-1 expression (Figure 5.2.4 b), NaHS significantly inhibited surface VCAM-1 expression (1000 μM, % inhibition 42.95 ± 5.43, n = 8, Figure 5.2.6 b). Interestingly, NaHS had a bi-phasic effect on surface VCAM-1 expression, where low concentrations also inhibited surface VCAM-1 expression (62.5 μM, % inhibition 54.00 ± 4.97, $P<0.05$) and middle concentrations 125 – 500 μM had no effect (Figure 5.2.6 b).

To ensure the adhesion molecule effects of GYY4137 or NaHS were due to the release of H_2S. These H_2S donors were solubilised in culture medium and utilised when H_2S was no longer detected (typically > 4 weeks for GYY4137 5 mM). Preliminary experiments (n = 2) indicate that time-expired GYY4137 and NaHS did not have an effect on surface VCAM-1 expression (Figure 5.2.7), indicating the effects of adhesion molecule expression are indeed through the release of H_2S from GYY4137 and NaHS.

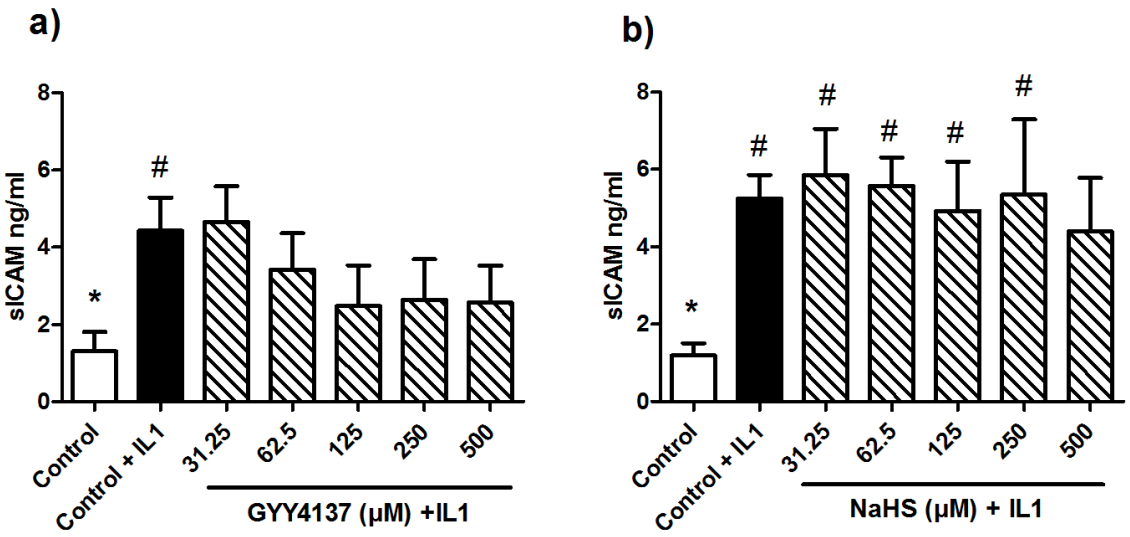

Figure 5.2.3. HUVEC were pre-treated with GYY4137 (31.25 – 500 µM) or NaHS (31.25 – 500 µM) for 1 h prior to stimulation with IL-1β (10 ng/ml) for 24 h. Cell culture medium was assayed for soluble ICAM-1 using a commercial ELISA. Results show soluble ICAM-1 ng/ml as mean ± SEM, n = 4, *P<0.05 c.f. control + IL-1β, #P<0.05 c.f. control.

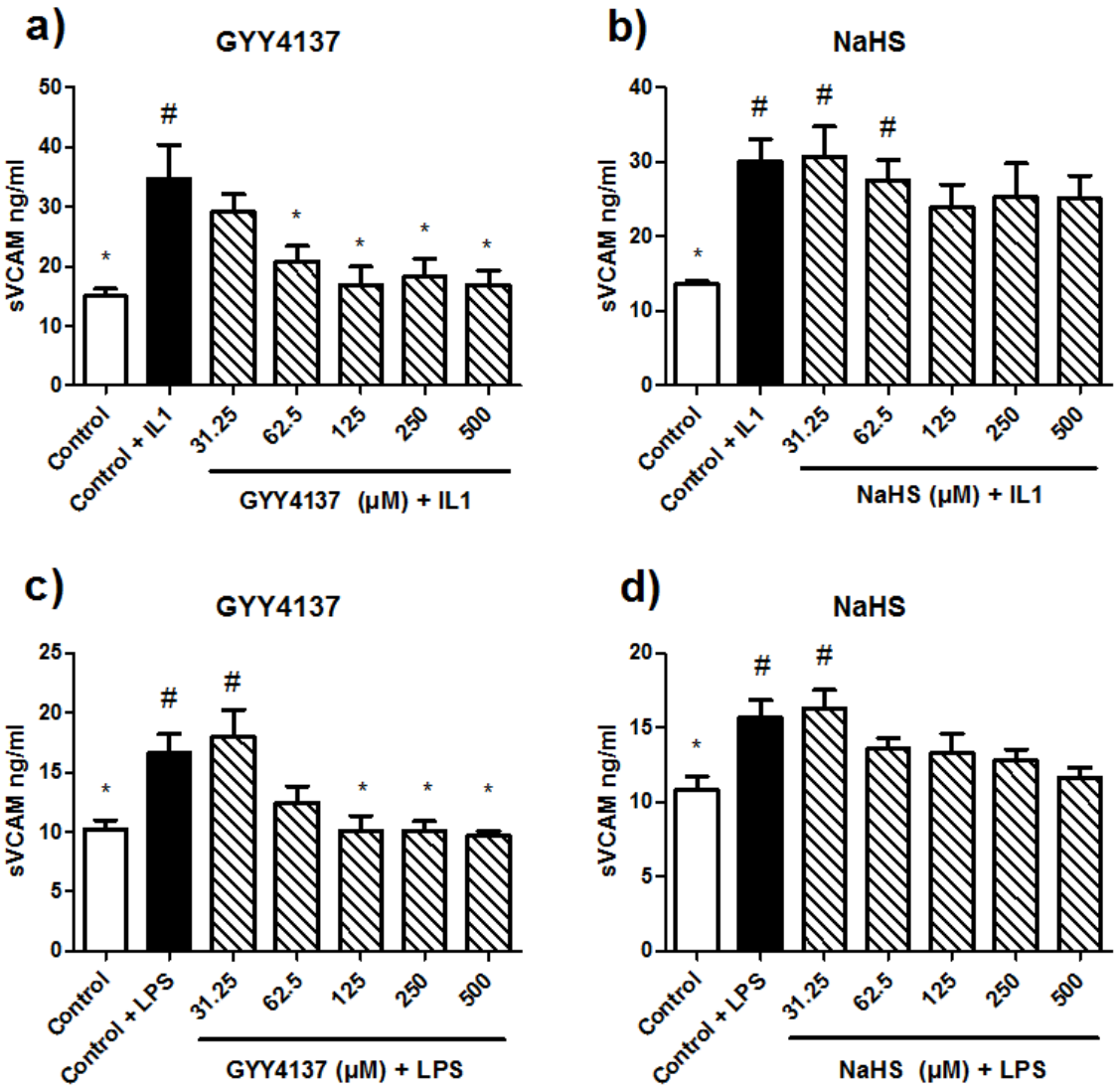

Figure 5.2.4. HUVEC were pre-treated with GYY4137 (31.25 – 500 μM) or NaHS (31.25 – 500 μM) 1 h prior to stimulation with IL-1β (10 ng/ml) or LPS (100 ng/ml). At 24 h cell culture medium was frozen and later used to measure soluble VCAM-1 using a commercial ELISA. GYY4137 pre-treatment significantly reduces soluble VCAM-1 expression induced by a) IL-1β or c) LPS. NaHS does not significantly reduce soluble VCAM-1 expression induced by b) IL-1β or d) LPS. Results show soluble VCAM-1 ng/ml as mean ± SEM, n = 4. *P<0.05 c.f. Control + IL-1β or LPS, #P<0.05 c.f. control.

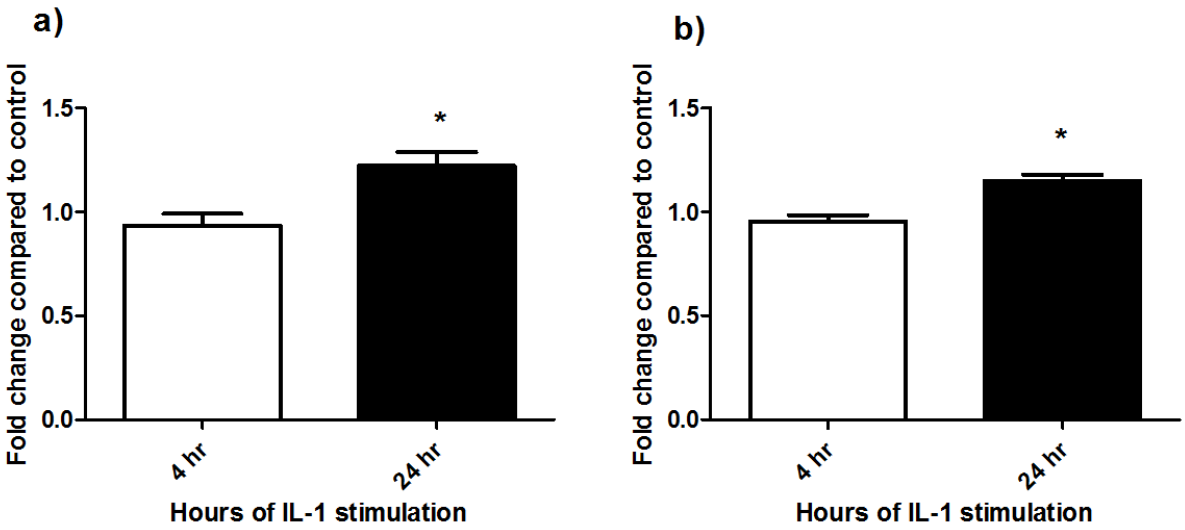

Figure 5.2.5. HUVEC were stimulated with IL-1β (10 ng/ml) for 4 h and 24 h. HUVEC were fixed and incubated with primary antibody against VCAM-1 or ICAM-1 and a secondary HRP-linked antibody. Surface adhesion molecule expression was quantitated through the HRP induced oxidation of TMB/H_2O_2 (OD 652 nm) and these values standardised to cell count (crystal violet assay, OD 610 nm). Data show fold change of control without IL-1β stimulation a) VCAM-1 b) ICAM-1. Results are expressed as fold change of control mean ± SEM, n = 3, *P<0.05 c.f. 4 h control.

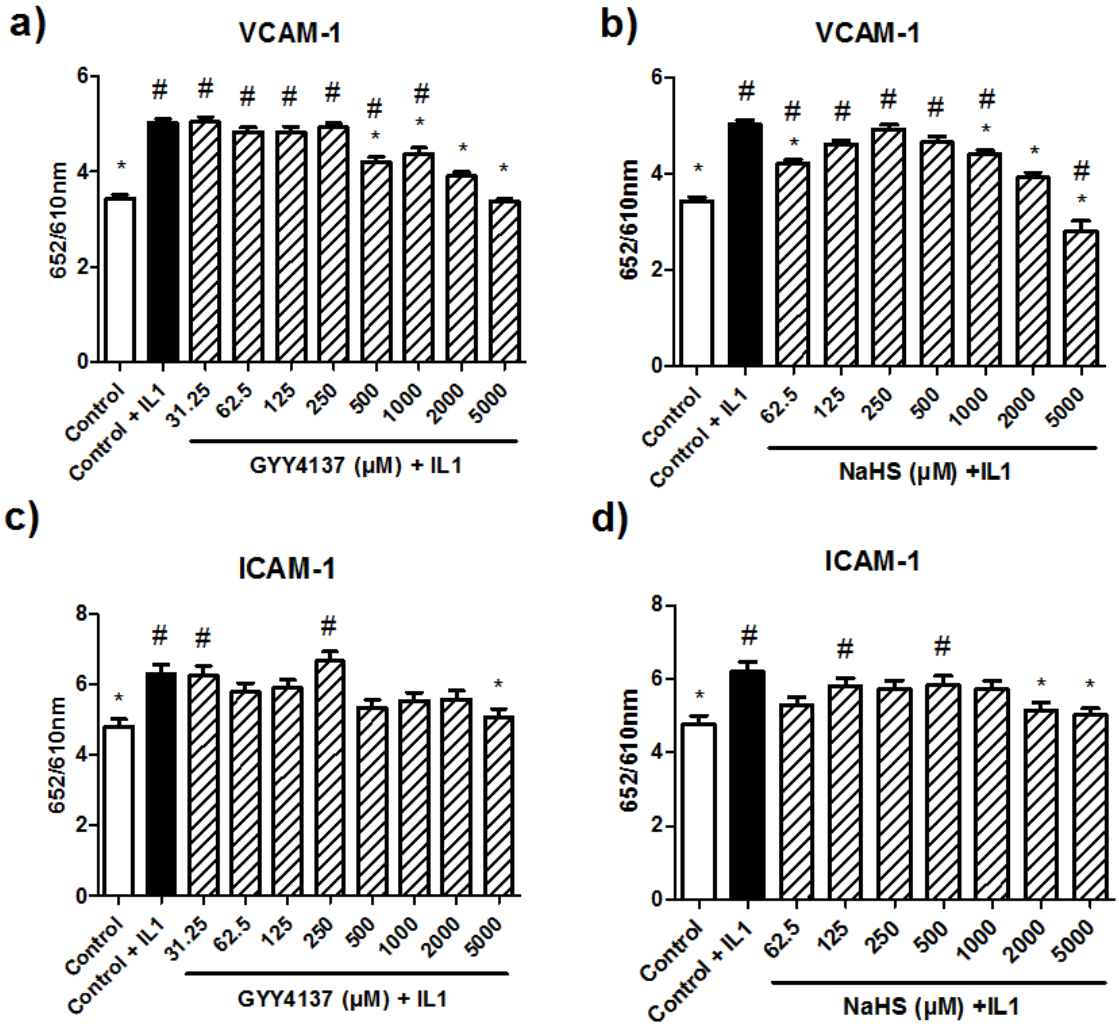

Figure 5.2.6. HUVEC were pre-treated with GYY4137 (31.25 – 5000 μM) or NaHS (62.5 – 5000 μM) 1 h prior to IL-1β (10 ng/ml) stimulation for 24 h. Cells were fixed and incubated with antibodies against VCAM-1 and ICAM-1, and a secondary HRP-linked antibody. Surface adhesion molecule expression was quantitated through the HRP induced oxidation of TMB/H_2O_2. Values were subsequently standardised to cell count, determined by the crystal violet assay. Surface VCAM-1 a) GYY4137 b) NaHS and surface ICAM-1 c) GYY4137 b) NaHS. Results show absorbance 652nm/610nm as mean ± SEM, n = 8, *P<0.05 c.f. Control + IL-1β, #P<0.05 c.f. control.

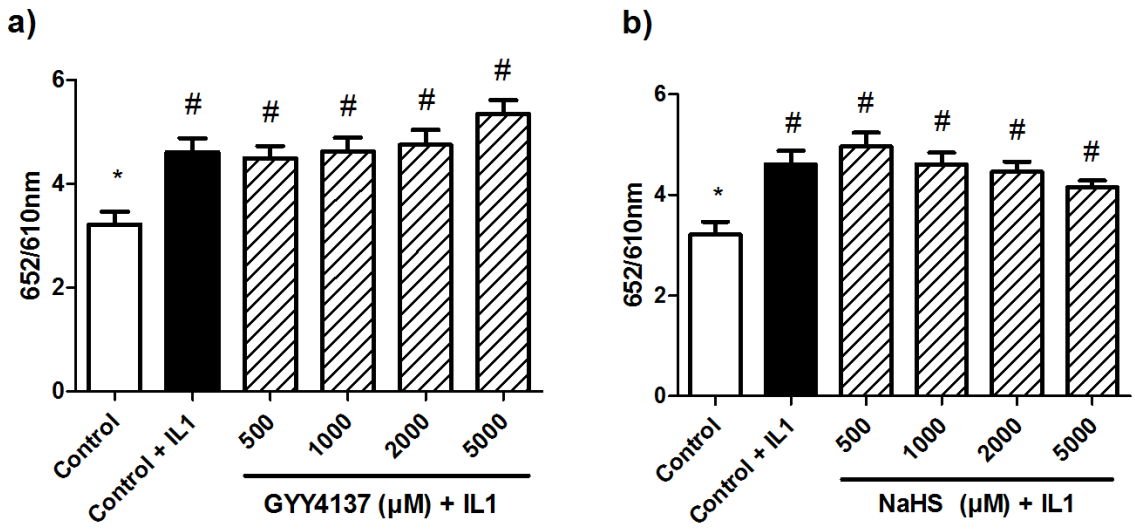

Figure 5.2.7. GYY4137 (500 – 5000 µM) and NaHS (500 – 5000 µM) were left to decompose and expire of H_2S by incubation at 37^{o}C > 4 weeks. HUVEC were pre-treated with time expired GYY4137 or NaHS for 1 h prior to stimulation with IL-1β (10 ng/ml) for 24 h. Cells were fixed and incubated with primary antibody against VCAM-1 and secondary HRP-linked antibody. VCAM-1 expression was quantitated through the HRP induced oxidation of TMB/H_2O_2. Values were subsequently standardised to cell count (crystal violet assay). Values show absorbance measured at 652nm/610nm mean ± SEM, n = 2 independent experiments (each in triplicate) *P<0.05 c.f. control + IL-1β, #P<0.05 c.f. control.

5.2.3. Effect of H_2S donors on HUVEC cell viability

To ensure the effects of H_2S on surface VCAM-1 was not due to cell death, two cell viability assays were performed. The crystal violet viability assay was carried out as an indicator of cell density and the ATP viability assay was undertaken to ensure the cells were still metabolically active.

Apoptosis is induced on serum starving HUVEC (Karsan, et al. 1997). As a positive control for the cell viability assays, HUVEC were treated in low (1% v/v) or normal (20% v/v) serum medium for 24 h. Cells grown in 20% v/v serum had a greater cell density (1.35 ± 0.02 fold increase, n = 4, $P<0.05$) compared to those grown in 1% v/v serum (Figure 5.2.8 b). Moreover, HUVEC grown in 20% v/v serum exhibited greater ATP production than those grown in 1% v/v serum (1.79 ± 0.16 fold increase, $P<0.05$, n = 4, Figure 5.2.8 a).

Cell viability assays were performed using the same protocol for measuring adhesion molecule expression in HUVEC. Consequently, HUVEC were pre-treated with GYY4137 (32.25 – 5000 µM) or NaHS (62.5 – 5000 µM) prior to stimulation for with IL-1β (10 ng/ml) for 24 h. GYY4137 did not significantly reduce cell metabolic activity (Figure 5.2.9 a) or cell density (Figure 5.2.10 a), suggesting the effects of GYY4137 on surface adhesion molecule expression was not as a result of cell death. Similarly, NaHS did not significantly reduce metabolic activity of HUVEC (Figure 5.2.9 b). However, NaHS (1 mM and 5 mM, $P<0.05$) significantly reduced HUVEC cell density (Figure 5.2.10 b).

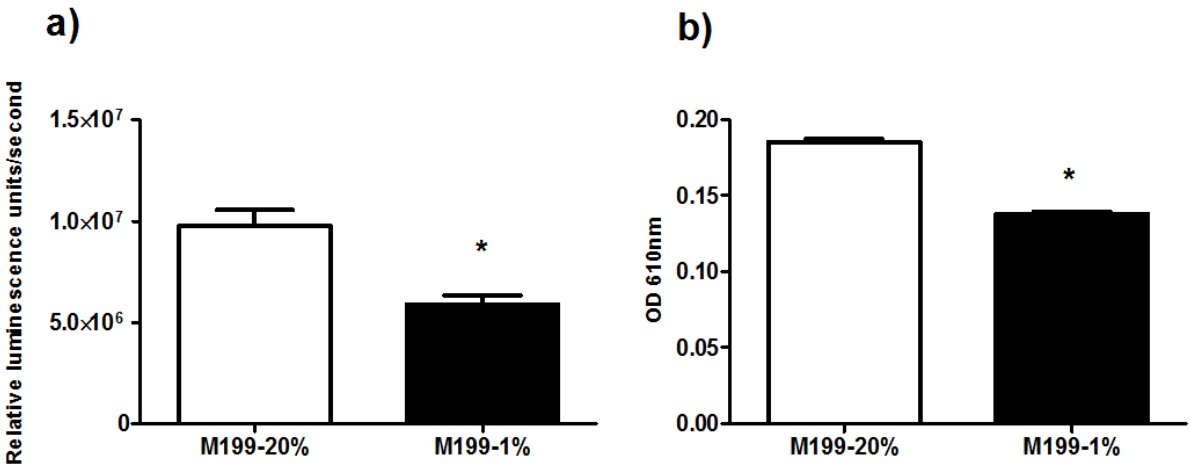

Figure 5.2.8. HUVEC were incubated in M199 containing 1% v/v (M199-1%) or 20% v/v (M199-20%) serum for 24 h. Incubating HUVEC in M199-1% significantly reduces cell viability compared to incubation in M199-20% determined by a) ATP metabolic viability assay, measured as relative luminescence units/second expressed as mean ± SEM, n = 4, *P<0.05 c.f. M199-20% b) crystal violet cell count assay, measured by absorbance at 610 nm and expressed as mean ± SEM, n = 4, *P<0.05 c.f. M199-20%.

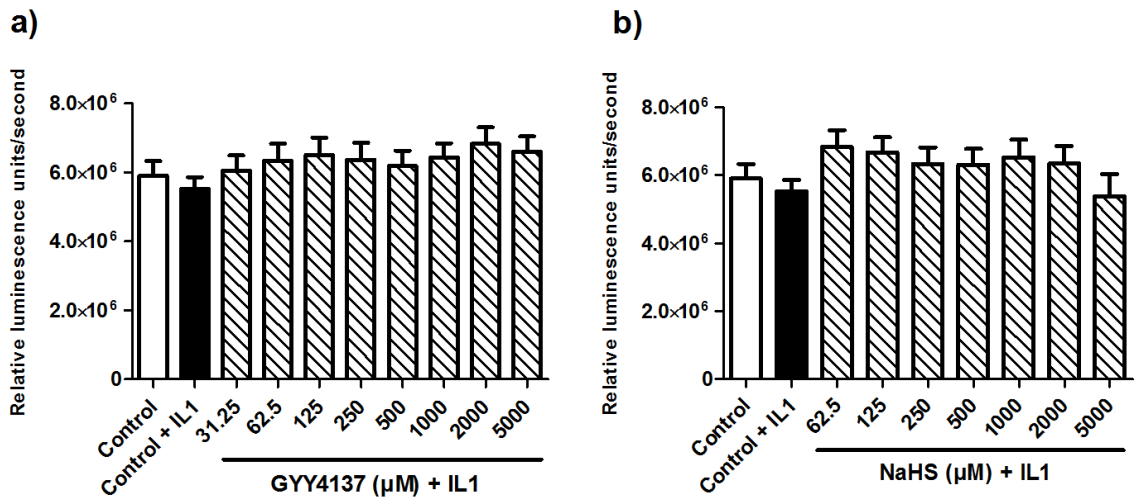

Figure 5.2.9. HUVEC were pre-treated with a) GYY4137 (31.25 – 5000 µM) or b) NaHS (62.5 – 5000 µM) for 1 h, prior to IL-1β (10 ng/ml) stimulation. At 24 h ATP was measured as an endpoint for cell viability using a commercially available kit. Results show relative luminescence units/second and expressed as mean ± SEM, n = 4.

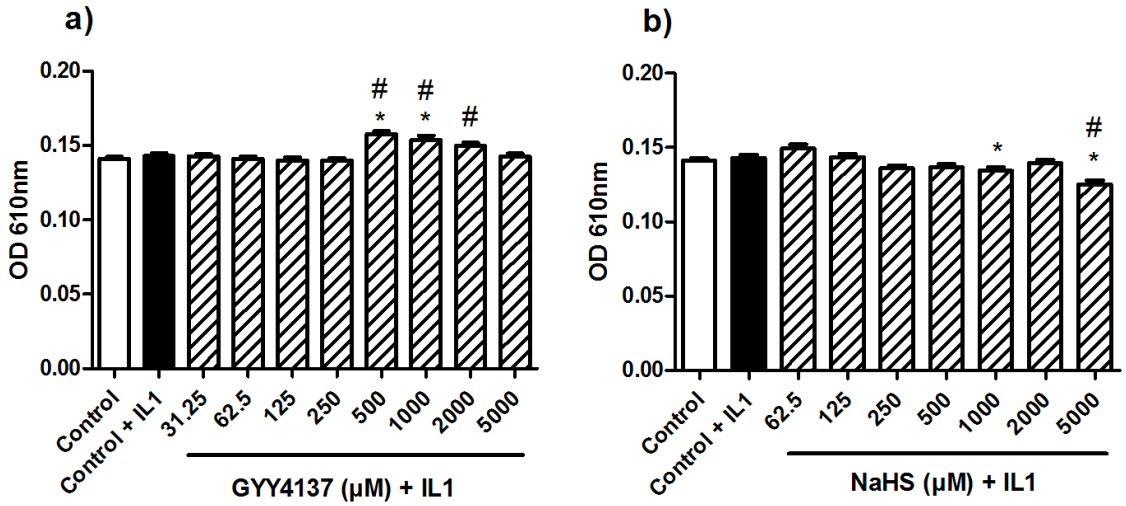

Figure 5.2.10. HUVEC were pre-treated with a) GYY4137 (31.25 – 5000 µM) or b) NaHS (62.5 – 5000 µM) for 1 h, prior to IL-1β (10 ng/ml) stimulation for 24 h. Cells were fixed and stained with crystal violet as an indicator of cell density. Results show absorbance at 610 nm, expressed as mean ± SEM, n = 8, *P<0.05 c.f. Control + IL-1β, #P<0.05 c.f. control.

5.2.4. Mechanism underlying the adhesion molecule effects of GYY4137

It has previously been reported that H_2S may be involved in regulating the NF-κB translocation pathway. To explore the mechanism behind the effect of GYY4137 on adhesion molecule expression, HUVEC were pre-treated with GYY4137 and stimulated with IL-1β. Subsequently, the cells were lysed and blotted for inhibitor of κB (IκB-α) and cyclooxygenase-2 (COX-2), two proteins up- and down-stream NF-κB respectively.

Western blots for IκB-α protein expression were carried out in HUVEC pre-treated with GYY4137 (125 μM) for 1 h prior to stimulation with IL-1β (10 ng/ml) for 15, 30, 60 and 120 min. GYY4137 (125 μM) reduced soluble VCAM-1 induced by both IL-1β and LPS (Figure 5.2.4) and was therefore the concentration used in this experiment.

Under normal conditions, IκB-α is found in the cytoplasm bound to NF-κB. Following a pro-inflammatory stimulus such as IL-1β stimulation, IκB-α is thought to be immediately ubiquitinated and degraded, therefore allowing NF-κB translocation to the nucleus (Karin 1999). In the absence of IL-1β stimulation (control cells), IκB-α protein was present in HUVEC (Figure 5.2.11 and 5.2.12). IL-1β treatment for 15 and 30 min did not lead to detectable amounts of IκB-α protein (Figure 5.2.11), suggesting IκB-α protein had been degraded following IL-1β stimulation. IκB-α was shown to be fully recovered by 120 min of IL-1β treatment (Figure 5.2.11). Preliminary studies suggested that pre-treating HUVEC with GYY4137 (125 μM) increased the rate of IκB-α recovery at 60 min (Figure 5.2.11, blot 2), suggesting that GYY4137 inhibited NF-κB translocation to the nucleus. However, on further work a lot of variation was noted in the recovery of IκB-α between HUVEC derived from different mothers (Figure 5.2.11). Blots 2 – 5

(Figure 5.2.11) suggest that GYY4137 (125 μM) increases the rate of recovery of IκB-α at 60 min compared to IL-1β alone without GYY4137 pre-treatment. However, blots 6 and 7 (Figure 5.2.11) showed that IκB-α did not recover at 60 min on GYY4137 pre-treatment, suggesting a later time point is necessary to determine whether GYY4137 induces a faster rate of IκB-α recovery, in these particular cells. In contrast, blots 8 – 10 (Figure 5.2.11) showed recovery of IκB-α by 60 min in both IL-1β control and GYY4137 pre-treated HUVEC potentially suggesting an earlier time point between 30 and 60 min would be necessary to determine if IκB-α in GYY4137 treated HUVEC was faster recovering. Pooling the data together suggested that GYY4137 (125 μM) pre-treatment did not increase the recovery rate of IκB-α protein (Figure 5.2.12 a) necessary to inhibit NF-κB translocation to the nucleus. Furthermore, increasing the concentration of GYY4137 (500 μM) did not improve the variation between HUVEC and the rate of IκB-α recovery was not significantly greater in the GYY4137 pre-treated HUVEC compared to the IL-1β control (Figure 5.2.12 b). Overall, this data suggest that, statistically, GYY4137 does not inhibit NF-κB translocation to the nucleus.

COX-2 expression, a protein downstream of NF-κB, was also used to determine whether GYY4137 inhibited NF-κB translocation to the nucleus. HUVEC were pre-treated with GYY4137 (125 – 500 μM) for 1 h prior to stimulation with IL-1β (10 ng/ml) for 24 h and western blots for COX-2 protein were carried out. IL-1β significantly enhanced the expression of COX-2 protein compared to control unstimulated HUVEC (Figure 5.2.13). Interestingly, GYY4137 (500 μM) pre-treatment significantly inhibited COX-2 expression in HUVEC (Figure 5.2.13). In contrast to the IκB-α data, this data suggested that GYY4137 inhibited NF-κB translocation to the nucleus.

iNOS is another protein downstream of NF-κB. Nitrate/nitrite production from LPS stimulated RAW264.7 cells is a robust technique utilised as an indicator of iNOS expression. As a result the therapeutic potential of both H_2S donors were further characterised using this technique. RAW264.7 cells were pre-treated with NaHS, GYY4137 and subsequently stimulated with LPS for 24 h and nitrite/nitrate was subsequently quantified. All compounds reduced nitrate/nitrite production from RAW264.7 cells in a concentration dependent manner (Figure 5.2.14).

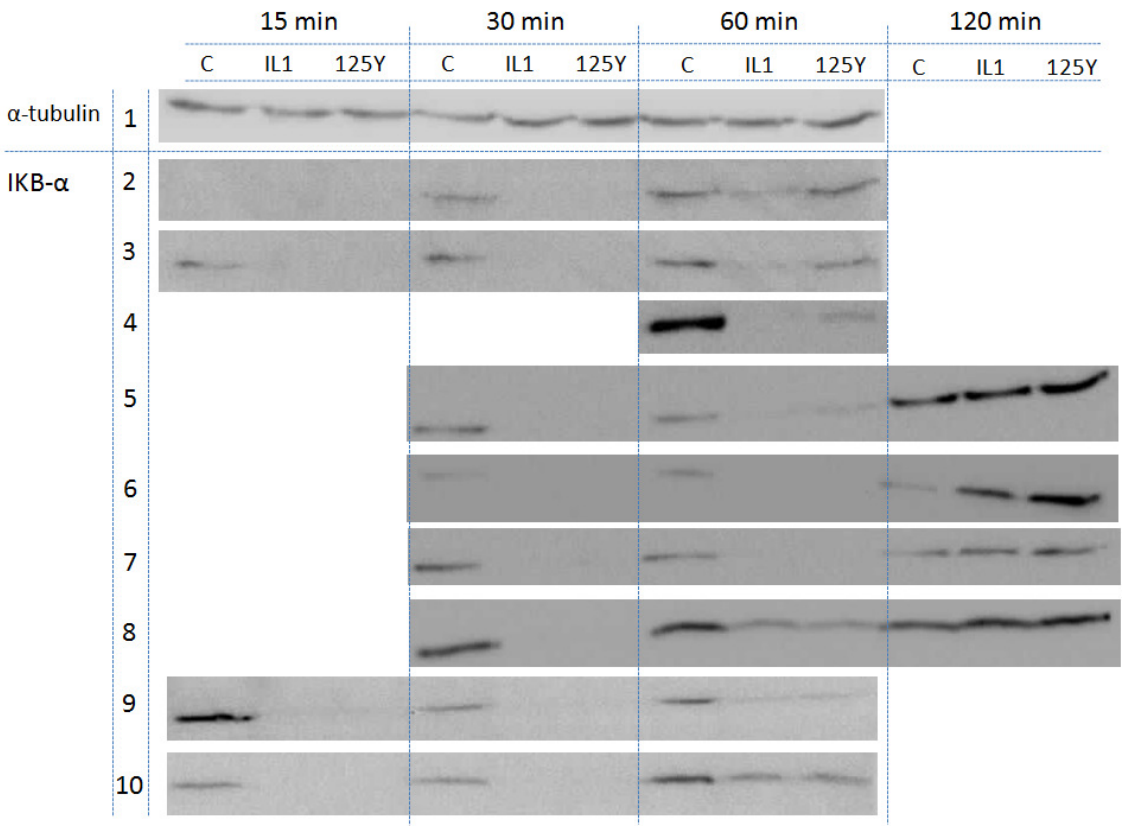

Figure 5.2.11. Western blots of IκB-α protein, n = 4-9, (blots 2 – 9, 43 kDa band found between molecular weight marker 35 – 50 kDa) from HUVEC pre-treated with GYY4137 (125 µM, denoted as 125 Y) and stimulated with IL-1β (10 ng/ml) for 15, 30, 60 or 120 min. Blot 1 is the loading control for blot 2 and represents a typical western blot for α-tubulin (52 kDa protein, found between molecular weight markers 50 – 75 kDa).

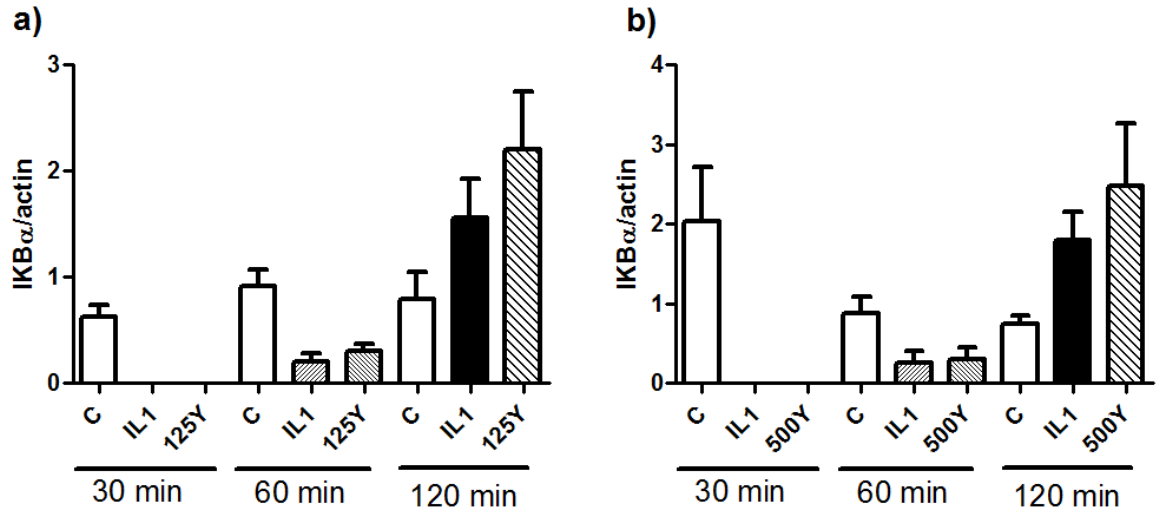

Figure 5.2.12. Column graphs showing IκB-α densitometry values standardised to α-tubulin loading control. HUVEC were pre-treated with a) GYY4137 125 µM where n = 4 – 9 (from blots 1 – 9 in Figure 5.2.11) or b) GYY4137 500 µM n = 4, prior to stimulation with IL-1β (10 ng/ml) for 30, 60 or 120 min. Results show mean ± SEM.

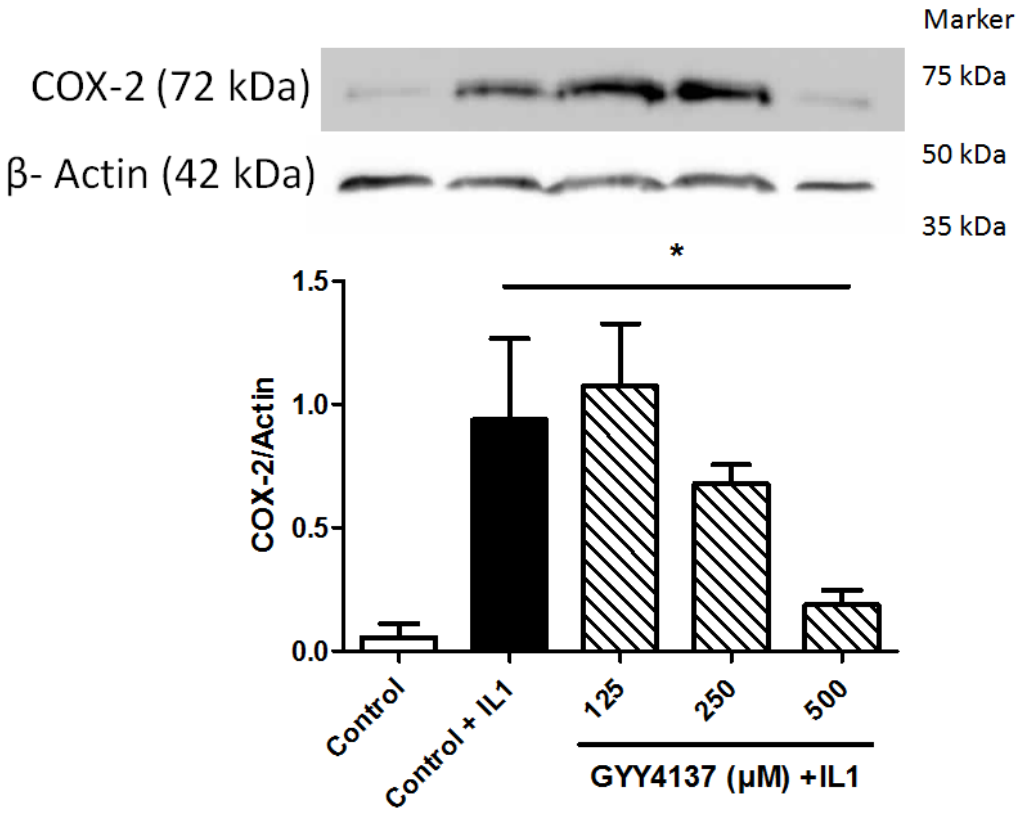

Figure 5.2.13. HUVEC were pre-treated with GYY4137 (125 – 500 μM) 1 h prior to stimulation with IL-1β (10 ng/ml) for 24 h. Western blots for COX-2 and β-actin (loading control) were made, and analysed by densitometry. Results show representative western blot and densitometry values of COX-2/β-actin shown as mean ± SEM, n = 3, *P<0.05 c.f. control + IL-1β

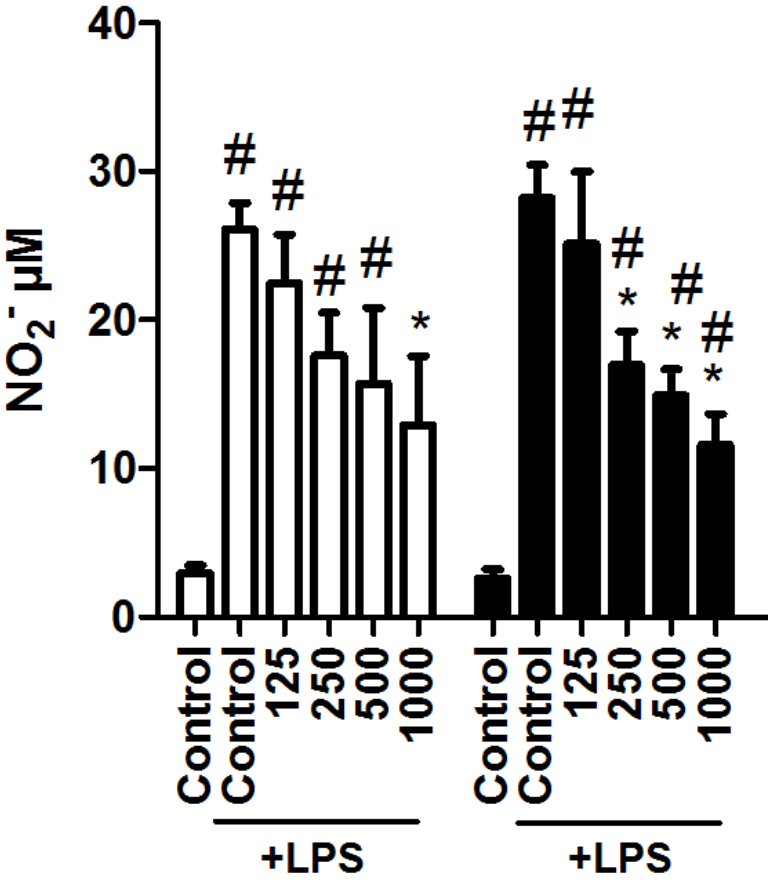

Figure 5.2.14. RAW 264.7 cells were pre-treated with NaHS (white bars) or GYY4137 (black bars), 125 – 1000 µM, for 1 h prior to stimulation with LPS (1 µg/ml) for 24 h. Culture medium was assayed for nitrite/nitrate using the Griess assay. Data show mean ± SEM, n = 5, #P<0.05 c.f. control, *P<0.05 c.f. control + LPS.

5.2.5. Carrageenan oedema hindpaw model characterisation

Carrageenan is a seaweed derivative and can induce local inflammation on subcutaneous injection (Campo, et al. 2009). The carrageenan hindpaw model was utilsed to characterise the effects of GYY4137 in this model of inflammation. It was first necessary to characterise endpoint markers in this model to assess appropriate time points to measure oedema, mechanical hyperalgesia and myeloperoxidase (MPO), prior to examining the effects of GYY4137 in this model. Carrageenan was injected into one hindpaw of anaesthetised mice and saline was injected into the contralateral paw.

At 0.5, 1.5, 3.5 and 5.5 h after carrageenan injection, hindpaw oedema was measured using a caliper. Carrageenan induced a significant increase in paw thickness at all time points (0.5 – 5.5 h) compared to saline control and this effect was greatest at 5.5 h (Figure 5.2.15). It should be noted that the small increase in paw thickness noted at 0.5 hs post-saline i.pl (and probably post-carrageenan) (Figure 5.2.15), is likely to result from an injection of a relatively large volume of fluid and is probably not indicative of a true inflammatory effect.

Mechanical hyperalgesia was carried out 30 min after measuring paw thickness (1, 2, 4 and 6 h post i.pl.). A Von Frey-type filament was used to measure mechanical hyperalgesia induced by carrageenan. The mechanical threshold effect of saline paws was significantly ($P<0.05$) lower than baseline at 1 h but recovered after 6 h (Figure 5.2.16 a). In contrast, the mechanical pain threshold in the carrageenan paws did not recover by 6 h (Figure 5.2.16 b).

On terminating the experiment, the hindpaws were cut and assayed for myeloperoxidase (MPO) activity (an indicator of leukocyte infiltration) or H_2S

synthesising enzyme activity. Saline i.pl injection did not induce a significant increase in MPO activity compared to naïve uninjected paws (Figure 5.2.17). In contrast, there was a significant increase in myeloperoxidase (MPO) activity in carrageenan paws compared to saline (Figure 5.2.16). No significant difference in MPO activity between carrageenan paws was noted at 4 and 6 h (Figure 5.2.16).

The injection of carrageenan into mouse hindpaws significantly ($P<0.05$) increased the endogenous H_2S synthesising activity compared to saline control at 6 h post- i.pl (Figure 5.2.18).

5.2.6. Effect of GYY4137 on pain and inflammation in the mouse hindpaw

To examine whether GYY4137 had an effect on paw oedema, mechanical hyperalgesia or MPO in carrageenan hindpaws GYY4137 (50 mg/kg i.p.) was injected 1 h post- i.pl. injection.

GYY4137 had no effect on paw oedema as determined by paw weight and paw thickness at 6 h (Figure 5.2.19). Moreover, GYY4137 did not significantly increase the pain threshold in the carrageenan paws at both 4 and 6 h after i.pl (Figure 5.2.20).

Carrageenan i.pl significantly ($P<0.05$) increased MPO activity compared to the saline i.pl injected contralateral paw, in both control and GYY4137 treated groups (Figure 5.2.21). However, comparing MPO activity between carrageenan injected paws of the two groups, the GYY4137 treated mice had significantly less MPO activity compared to control (% inhibition 55.46 ± 3.89, $P<0.05$, Figure 5.2.21).

Interestingly, incubating purified MPO with GYY4137 (5 μM) *in vitro* significantly reduced MPO enzyme activity (Figure 5.2.22) suggesting a direct interaction of GYY4137 with MPO, independent of leukocyte infiltration.

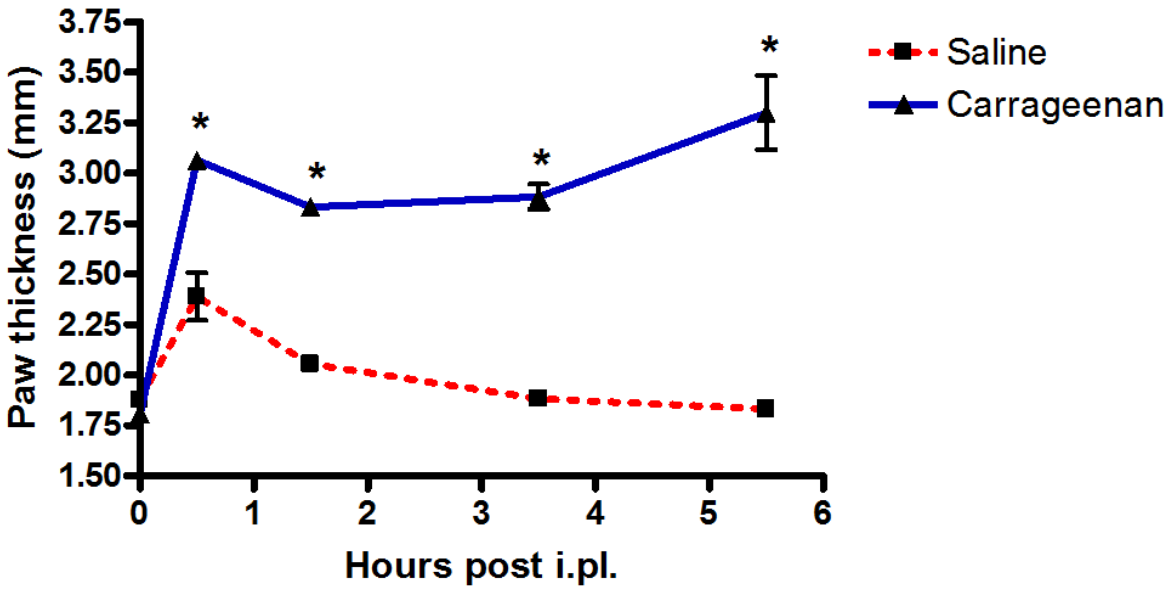

Figure 5.2.15. Mice were anaesthetised (isofluorane) and each hindpaw was injected intraplanter (i.pl) with either saline (50 µl) in one paw or carrageenan (50 µl, 1% w/v) in the contralateral paw. Paw thickness was measured using a calliper at 0.5 h, 1.5 h, 3.5 h and 5.5 h. Results show paw thickness (mm) shown as mean ± SEM over time, n = 6, *P<0.05 c.f. saline time control.

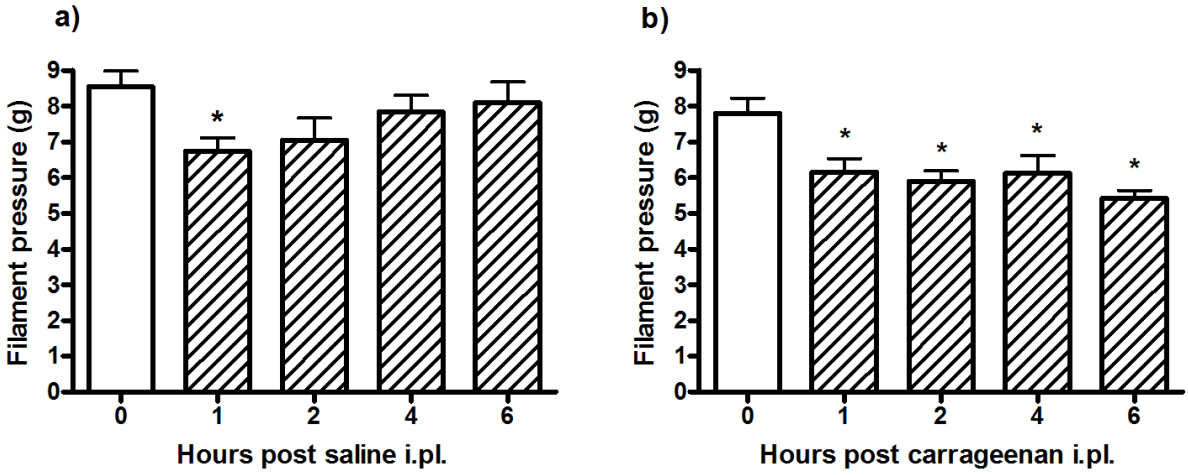

Figure 5.2.16. Mice were anaesthetised (isoflurane) and each hindpaw was injected intraplanter (i.pl) with either a) saline (50 µl) in one paw or b) carrageenan (50 µl, 1% w/v) in the contralateral paw. A plantar aesthesiometer attached to a Von Frey-type filament was used to measure paw withdrawal reflex at 0 h, 1 h, 2 h, 4 h and 6 h post i.pl. Data show force (g) at which paw was withdrawn, shown as mean ± SEM, n = 6, *P<0.05 c.f. time 0 h.

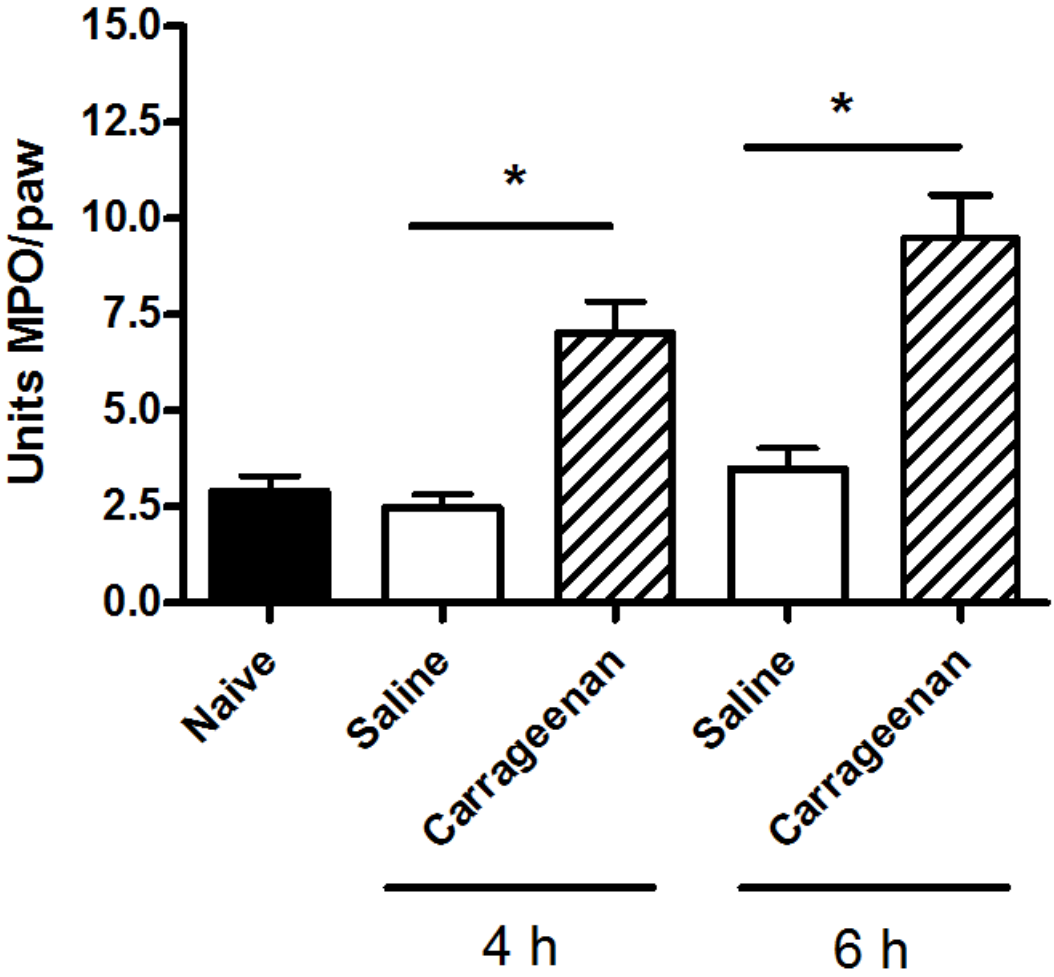

Figure 5.2.17. The hindpaws of mice was injected intraplanter (i.pl) with either saline (50 µl) in one paw or carrageenan (50 µl, 1% w/v) in the contralateral paw. Mice were culled at 4 h or 6 h after i.pl and hindpaws frozen. Myeloperoxidase (MPO) activity was assayed in mouse hindpaws with no injection (naïve), saline or carrageenan injection. Results show MPO units/paw shown as mean ± SEM, n = 6, *P<0.05 saline c.f. carrageenan

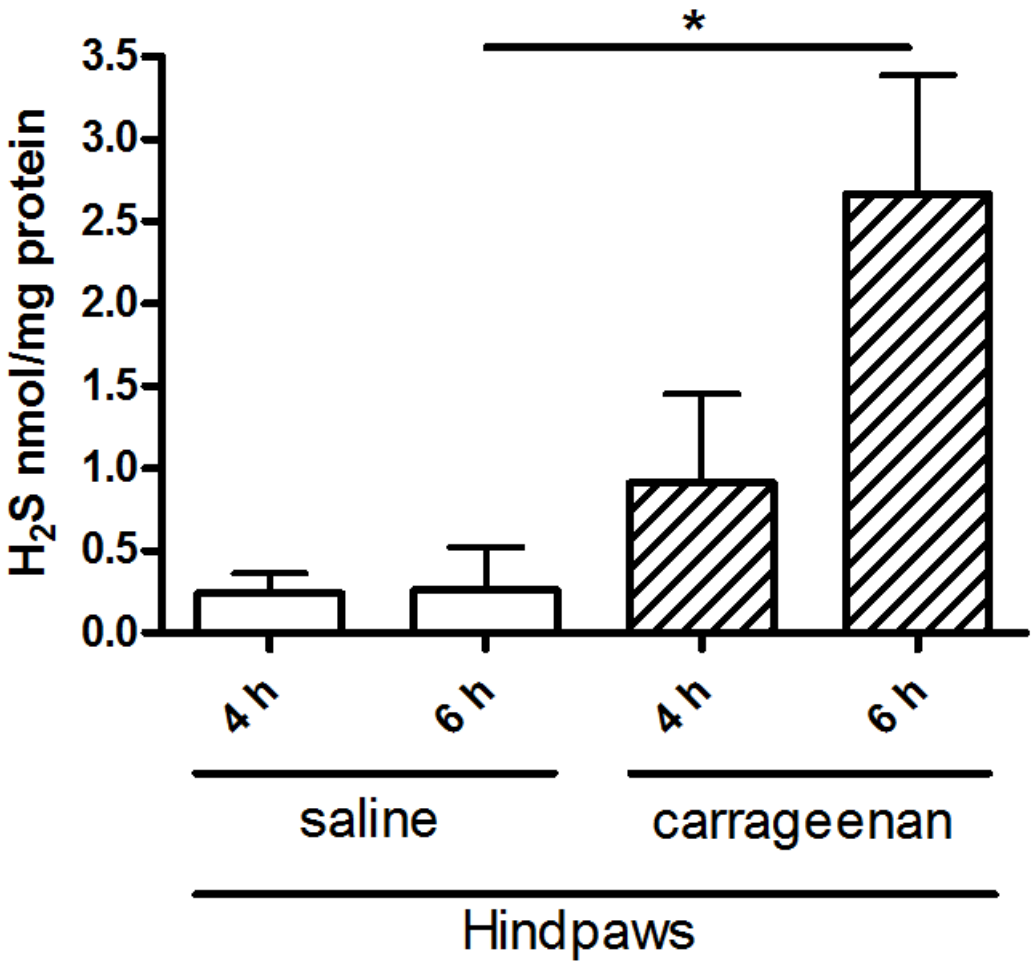

Figure 5.2.18. The hindpaws of mice was injected intraplanter (i.pl) with either saline (50 µl) in one paw or carrageenan (50 µl, 1% w/v) in the contralateral paw. Mice were culled at 4 h or 6 h after i.pl and hindpaws frozen. H_2S synthesising enzyme activity assay was carried out in the hindpaw homogenates. H_2S (µM) produced was measured using the methylene blue assay and standardised to protein, as an indicator of H_2S synthesising enzyme activity. Results show H_2S nmol/mg protein shown as mean ± SEM, n = 3, *P<0.05 c.f. saline control

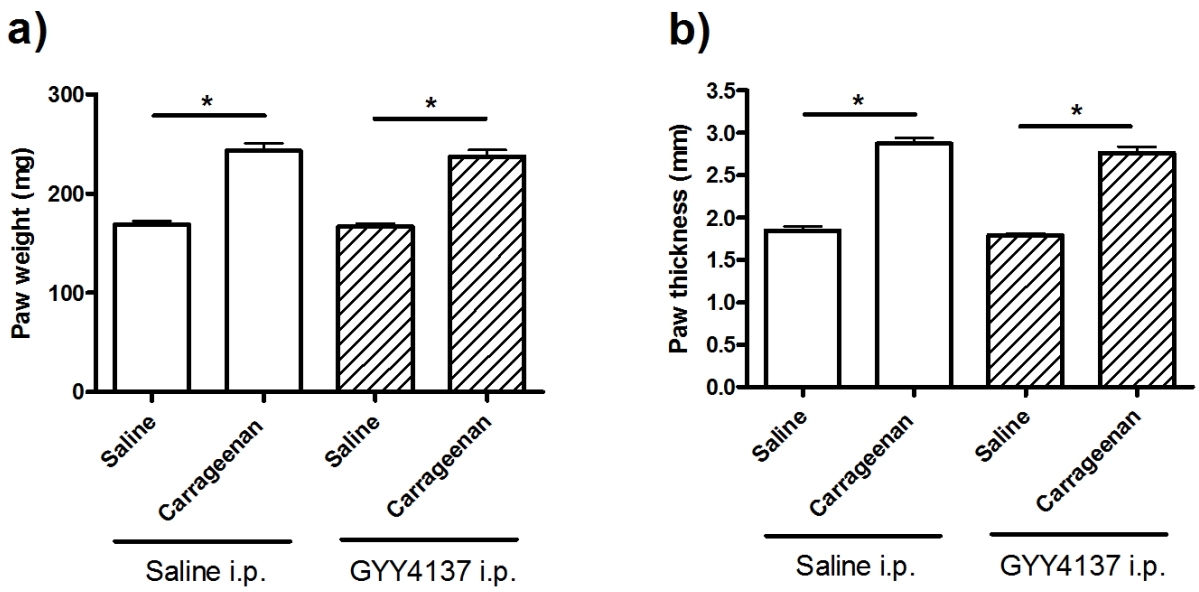

Figure 5.2.19. The hindpaws of mice was injected intraplanter (i.pl) with either saline (50 µl) in one paw or carrageenan (50 µl, 1% w/v) in the contralateral paw. An intraperitoneal (i.p.) injection of GYY4137 (50 mg/kg) or saline (10 ml/kg) was given 1 h post i.pl injection. Paramaters for oedema formation was measured a) paw weight at 6 h b) paw thickness at 5.5 h. Results show paw weight (mg) or paw thickness (mm) shown as mean ± SEM, n = 9, *P<0.05 saline c.f. carrageenan control

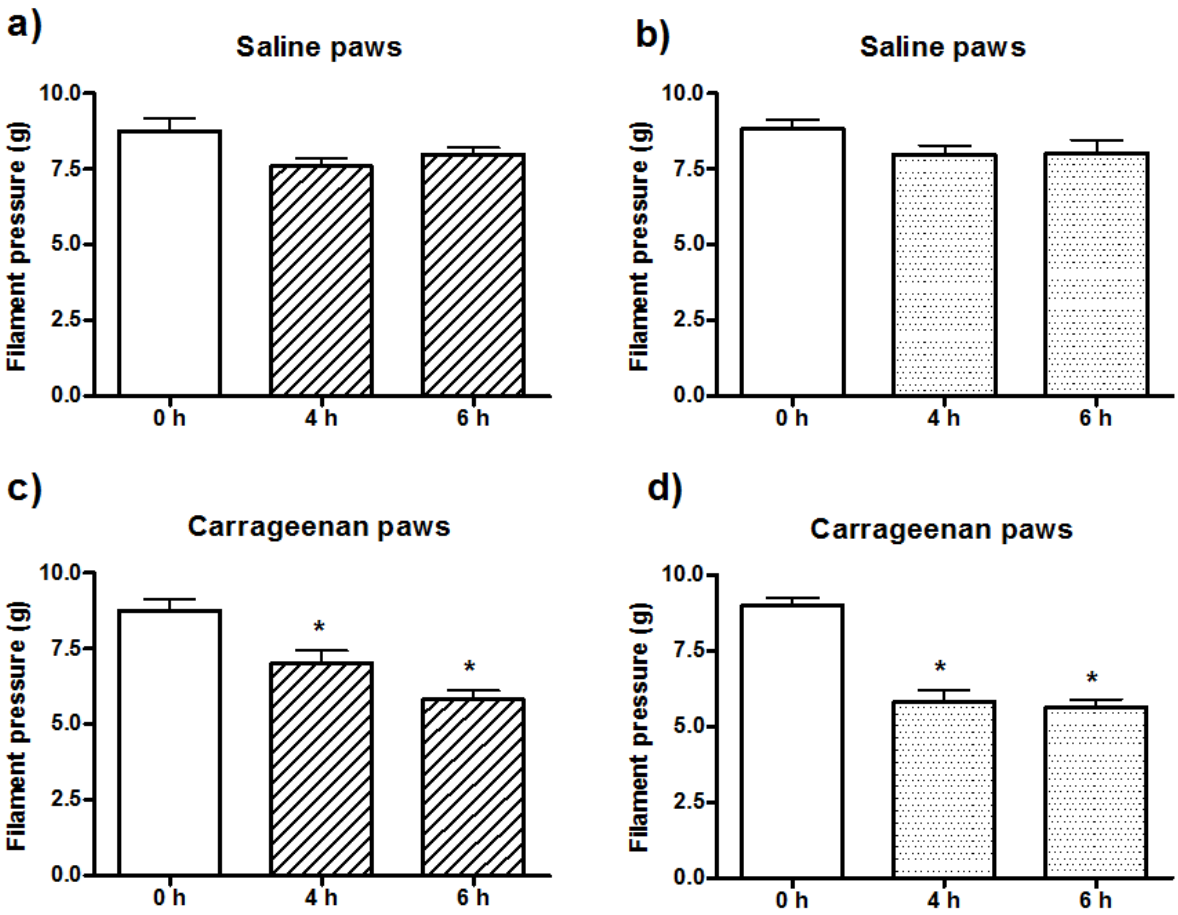

Figure 5.2.20. The hindpaws of mice was injected intraplanter (i.pl) with either saline (50 µl) in one paw or carrageenan (50 µl, 1% w/v) in the contralateral paw. An intraperitoneal (i.p.) injection of a) and c) saline (10 ml/kg) or b) and d) GYY4137 (50 mg/kg) was given 1 h post i.pl injection. A plantar aesthesiometer attached to a Von Frey-type filament was used to measure paw withdrawal reflex at 4 h and 6 h post i.pl. Data show filament pressure (g) at which paw was withdrawn, shown as mean ± SEM, n = 9, *P<0.05 c.f. time 0 h.

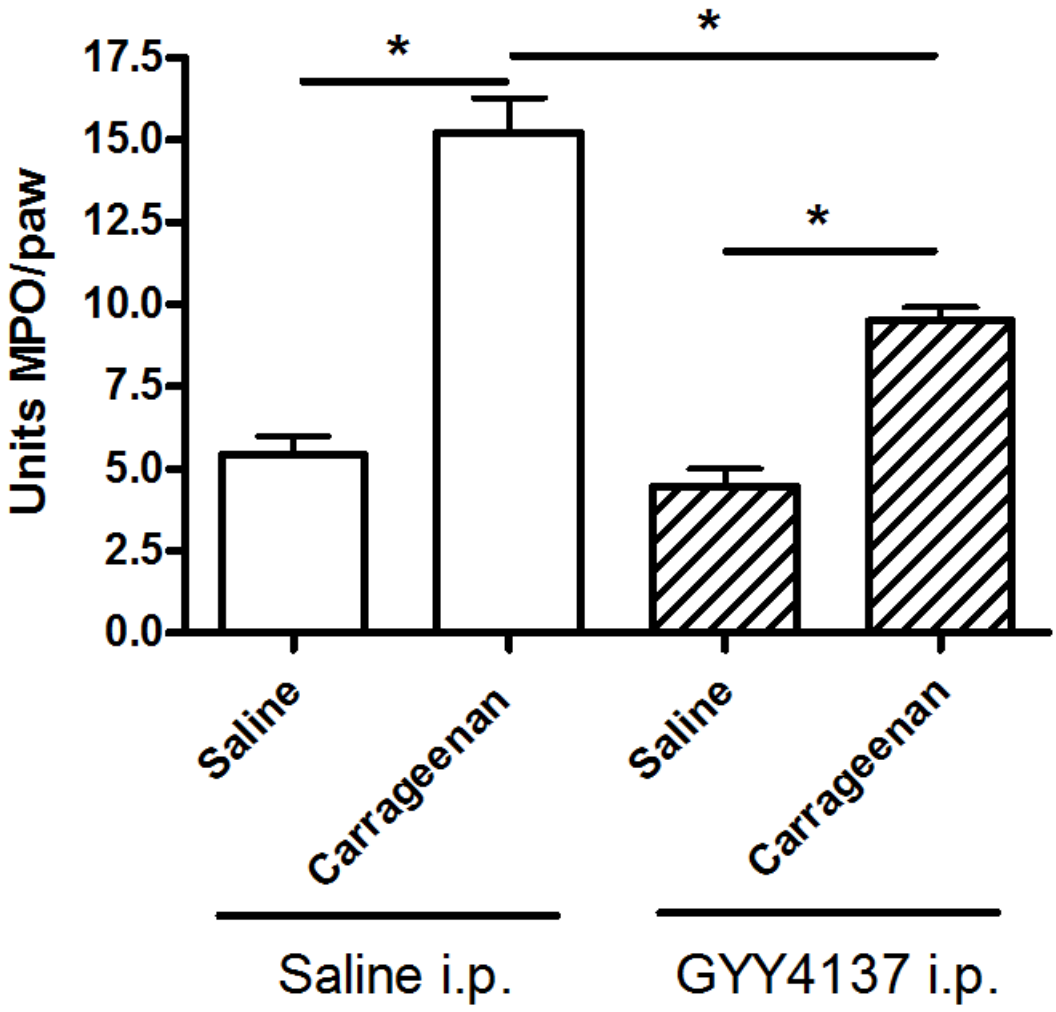

Figure 5.2.21. The hindpaws of mice was injected intraplanter (i.pl) with either saline (50 µl) in one paw or carrageenan (50 µl, 1% w/v) in the contralateral paw. An intraperitoneal (i.p.) injection of GYY4137 (50 mg/kg) or saline (10 ml/kg) was given 1 h post i.pl injection and mice were culled at 6 h. Myeloperoxidase (MPO) activity was assayed in hindpaws. GYY4137 significantly reduced carrageenan induced MPO activity. Data show units/MPO per paw, expressed as mean ± SEM, n = 6, *P<0.05

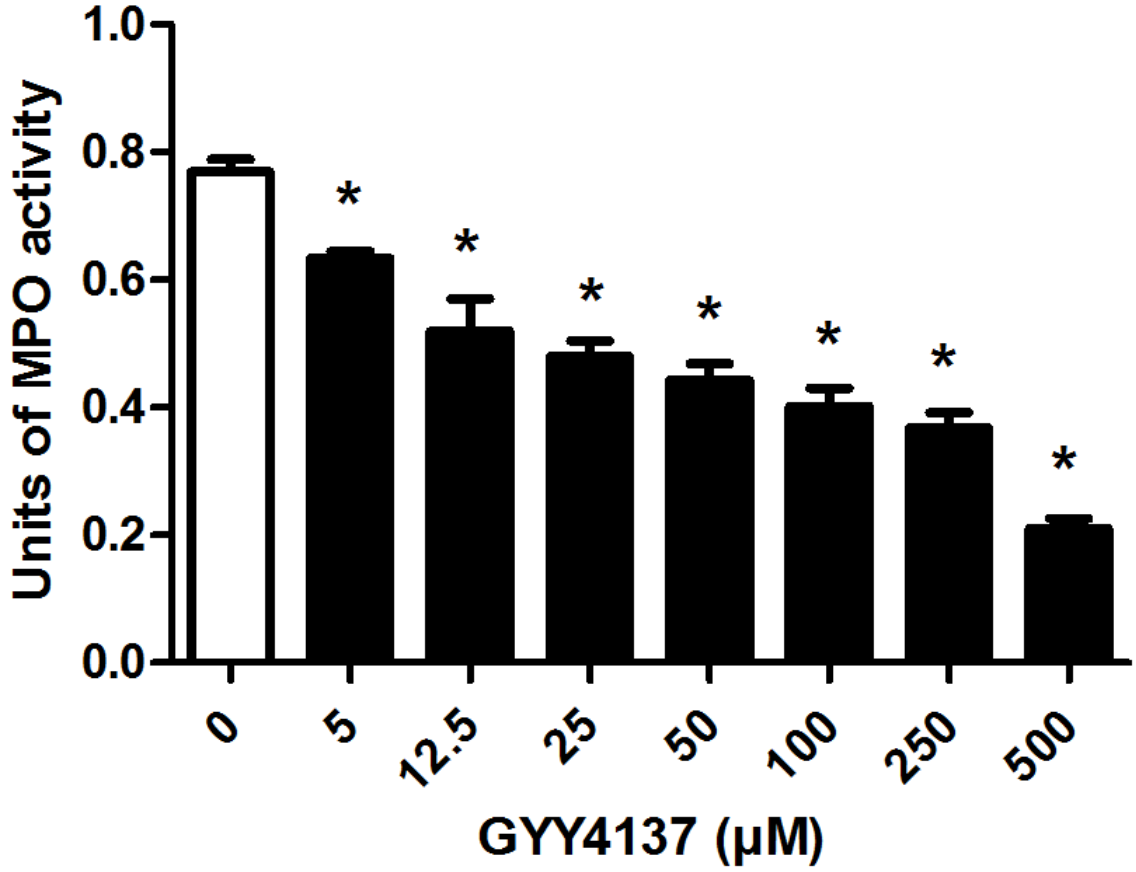

Figure 5.2.22. Purified human myeloperoxidase (MPO, 0.75 units/ml) was incubated with GYY4137 (5 – 500 µM) for 1 h at 37^{o}C. MPO enzyme activity was measured through the oxidation of TMB. Results are units of MPO activity shown as mean ± SEM, n = 4, *P<0.05 c.f. 0.

5.3. Discussion

The main findings from this chapter are:

1. GYY4137 and NaHS are capable of reducing VCAM-1 and ICAM-1 expression in HUVEC
2. Unlike NaHS, GYY4137 is unlikely to have cytotoxic effects at the concentrations utilised in HUVEC
3. The adhesion molecule effects of GYY4137 is perhaps, through inhibiting NF-κB translocation to the nucleus
4. GYY4137 appears to have anti-inflammatory effects *in vivo*, as demonstrated by a reduction in MPO activity in carrageenan hindpaws

Overall, GYY4137 and NaHS are capable of reducing VCAM-1 and ICAM-1 expression from HUVEC but not E-selectin; in contrast to NaHS, the effects of GYY4137 on adhesion molecule expression are unlikely to result from cell death. In correlation with the adhesion molecule data, GYY4137 appears to reduce MPO activity in carrageenan hindpaws. However, GYY4137 does not have an effect on oedema or mechanical hyperalgesia.

5.3.1. H_2S and adhesion molecule expression

To reach the site of inflammation, leukocytes must migrate from the blood into the tissues. To accomplish this, leukocytes need to attach and adhere to the endothelial lining along the vessel wall and subsequently migrate through this endothelial barrier and its underlying basement membrane to the affected tissues. The process of

leukocyte attachment and tethering/rolling along the endothelial layer is aided by selectins e.g. P-selectin or E-selectin, and the process of leukocyte arrest is assist by adhesion molecules such as ICAM-1/VCAM-1 (Ley, et al. 2007).

This chapter demonstrates that both H_2S donors, NaHS and GYY4137 are capable of reducing VCAM-1, ICAM-1 but not soluble E-selectin expression in IL-1β treated HUVEC suggesting H_2S donors may be capable of inhibiting leukocyte firm adhesion but not rolling along the endothelium. The effects on adhesion molecule expression are likely to result from H_2S released from GYY4137 or NaHS rather than the parent molecule itself as decomposed GYY4137 or NaHS had no effect on adhesion molecule expression (Figure 5.2.7). Moreover the effects of GYY4137 (32.5 – 5000 μM) on adhesion molecule expression were not as a consequence of a cell death, determined by cell density (crystal violet) and cell metabolism (ATP endpoint). In correlation with the current study, previous studies have also demonstrated that GYY4137 (100 μM) does not induce cell death in rat aortic vascular smooth muscle cells as measured by the trypan blue exclusion assay and p53 expression (Li, et al. 2008b). However, NaHS (1 mM) in the current study significantly ($P<0.05$) reduced HUVEC cell density (Figure 5.2.10) and may be one reason why NaHS reduced surface VCAM-1 (1 mM, $P<0.05$) and ICAM-1 (2 mM, $P<0.05$) expression at this concentration (Figure 5.2.6). However, it should be noted that adhesion molecule expression in the current study has been standardised to cell density, which may suggest that irrespective of the cell density, NaHS is capable of reducing adhesion molecule expression on HUVEC. Interestingly, both H_2S donors appeared to increase ATP production from HUVEC (Figure 5.2.9, not significant). H_2S has been shown to inhibit cytochrome *c* oxidase activity in mitochondria *in vitro* (Beauchamp, et al. 1984) and

therefore a reduction in cell ATP generation may be expected. The increase in ATP production observed in this study may be due to: a compensatory increase in ATP production by glycolysis, as a result from the reduction of ATP by inhibition of the oxidative phosphorylation (Leschelle, et al. 2005); H_2S has also been suggested to contribute to the donation of electrons to the electron transport chain and may be an alternative reason why an increase in ATP was noted (Goubern, et al. 2007). Whether the increase in ATP described in the current study is as a result of an increased activity by oxidative phosphorylation or gylcolysis requires further examination.

Although the effects of NaHS on adhesion molecule expression may be due to cell death at higher millimolar concentrations, interestingly, NaHS had a biphasic effect on surface VCAM-1 expression. Relatively low concentrations of NaHS ~60 μM and high millimolar concentrations of NaHS (1 – 5 mM) significantly ($P<0.05$) reduced surface VCAM-1 expression. However concentrations between NaHS 100 – 1000 μM had no significant effect (Figure 5.2.6 b). Interestingly, another study using NaHS (50 – 1000 μM) demonstrated a biphasic effect on PGE_2 and nitrite production from LPS stimulated RAW 264.7 cells (Whiteman, et al. 2010c). In the latter study, NaHS (50 – 100 μM) decreased PGE_2 and nitrite production whilst NaHS (200- 1000 μM) increased the production of these inflammatory mediators (Whiteman, et al. 2010c). These biphasic effects on inflammatory mediators also occur with $^{\bullet}NO$ donors. $^{\bullet}NO$ donor DEA-NO (30 nM – 300 μM, 24 h) was previously demonstrated to induce a biphasic effect on iNOS and COX-2 protein expression (Connelly, et al. 2001). In the latter study DEA-NO (300 nM to 3 μM) significantly increased iNOS and COX-2 protein expression in LPS stimulated RAW 264.7 cells (Connelly, et al. 2001). However, DEA-NO (30 nM, 30 - 300 μM) had no effect or significantly reduced iNOS and COX-2 expression (Connelly,

et al. 2001). This effect of DEA-NO was suggested to be as a consequence of a biphasic regulation of NF-κB activity (Connelly, et al. 2001), and may also be a mechanism by which NaHS induces its effects.

In correlation with the current study, another study demonstrated that pre-treating HUVEC with NaHS (1 μM, 6 h) significantly inhibited ICAM-1 protein expression induced by TNF-α (10 ng/ml, 6 h) (Wang, et al. 2009b). More recently, NaHS (100 μM, 30 min pre-treatment, $P<0.05$) has been shown to significantly inhibit E-selectin, P-selectin, VCAM-1 and ICAM-1 mRNA expression induced by TNF-α (10 ng/ml, 6 h) (Pan, et al. 2011). However, in the current study neither NaHS nor GYY4137 (31.25 – 500 μM) pre-treatment significantly reduced soluble E-selectin protein expression from HUVEC (Figure 5.2.2). The reason behind the difference in E-selectin expression in the current study and the latter study may be due to the difference in stimulating agent (IL-1β c.f. TNF-α), time course (24 h c.f. 6 h), the methods used to quantitate E-selectin expression (ELISA c.f. RT-PCR) or the type of E-selectin itself (soluble E-selectin c.f. E-selectin message). Although, E-selectin mRNA is an indicator of protein expression, message is not always translated to protein and the latter study did not directly examine E-selectin protein expression (Pan, et al. 2011).

5.3.2. Mechanism behind the adhesion molecule effects of GYY4137 in vitro

NF-κB is an important transcription factor in the immune system involved in the regulation of cytokine, effector enzyme and growth factor expression (Hayden and Ghosh 2004). In the inactivated form, NF-κB exists in the cytosol as a hetero- or homodimer bound to IκB which prevents NF-κB from translocating to the nucleus (Hayden and Ghosh 2004). In the classical pathway of NF-κB activation, pro-

inflammatory mediators such as TNF-α, IL-1, LPS, result in downstream signalling pathways that lead to the phosphorylation of the β subunit of IκB kinase (IKK) causing its activation and the subsequent phosphorylation of two conserved amino acids (Ser32 and Ser36) on the N-terminal domain of IκB (Karin 1999, Hayden and Ghosh 2004). Phosphorylated IκBs subsequently undergo post-translational modification termed polyubiquitination (Karin 1999). When the target protein is conjugated to a polyubiquitin chain a multisubuinit protease complex, the 26S proteasome, catalyses the protein into small peptides (Hochstrasser 2009). The uninhibited NF-κB is then capable of translocating to the nucleus and bind to promoter and enhancer regions of DNA containing κB sites with consensus sequence GGGRNNYYCC (R = purine, Y = pyrimidine, N = any base) (Hayden and Ghosh 2004).

H_2S has been suggested to inhibit NF-κB translocation to the nucleus. H_2S donor NaHS has been demonstrated to increase the rate of IκB-α recovery (NaHS 10 μM, IL-1β 2.5 ng/ml, fibroblast-like synoviocytes (FLS) (Stuhlmeier, et al. 2009); NaHS 200 μM, LPS 1 μg/ml, RAW264.7 (Oh, et al. 2006)). NaHS has also been demonstrated to inhibit IκB-α phosphorylation (NaHS 2 mM, IL-1β 2.5 ng/ml, FLS (Stuhlmeier, et al. 2009); NaHS 0.2 mg/kg 10 min prior to retransfusion in a model of hemorrhagic shock in rats (Ganster, et al. 2010)). NaHS can also directly inhibit NF-κB translocation into the nucleus (NaHS 200 μM, LPS 1 μg/ml, RAW264.7 (Oh, et al. 2006); NaHS 1000 μM, LPS 1 μg/ml, RAW264.7 (Whiteman, et al. 2010c)).

Conversely, NaHS has also been demonstrated to enhance NF-κB nuclear translocation, by mechanisms such as: inducing IκB-α degradation (NaHS 100 μM, U937 human monocyte cell line (Zhi, et al. 2007)); or directly enhance the translocation of NF-κB into the nucleus (NaHS 100 μM, U937 (Zhi, et al. 2007); NaHS 10

mg/kg i.p. at the time of cecal ligation operation (Zhang, et al. 2007b); NaHS 100-200 μM, LPS 1 μg/ml, RAW264.7 (Whiteman, et al. 2010c)). This evidence suggests that H_2S has variable roles in regulating the NF-κB translocation pathway.

Adhesion molecules VCAM-1, ICAM-1, and E-selectin have been shown to be regulated by the NF-κB pathway (Collins, et al. 1995), and was hypothesised as a mechanism by which GYY4137 may inhibit adhesion molecule expression. In the current study GYY4137 (125 μM or 500 μM 1 h pre-treatment) did not affect the rate of IκB-α recovery in HUVEC at 30 or 60 min induced by IL-1β 10 ng/ml (Figure 5.2.11 and 5.2.12), suggesting that GYY4137 may not be acting through inhibition of NF-κB translocation to the nucleus in these circumstances. Although it should be noted that the rate of IκB-α recovery appeared vary between HUVEC from different mothers (Figure 5.2.11). Indeed, the evidence for variation between HUVEC has previously been documented (Hofman, et al. 1993, Zoellner, et al. 1998, Overhoff and Sczakiel 2005, Paez, et al. 2008). Indeed further study to examine more time points for IKB-α degradation, or alternative means of measuring NF-κB translocation are necessary.

COX-2 is downstream NF-κB (Nie, et al. 2003), and has previously been used as an indicator of NF-κB translocation to the nucleus (Liang, et al. 1999). In contrast to the IκB-α data, GYY4137 (500 μM) significantly ($P<0.05$) reduced IL-1β induced COX-2 expression at 24 h in HUVEC (Figure 5.2.13), suggesting that GYY4137 inhibits NF-κB translocation. The variability between the rate of IκB-α recovery between HUVEC (30 – 120 min) in the current study made it difficult to interpret the data, particularly as only one time point (60 min) was used to determine IκB-α recovery. Endpoint markers such as the maximal expression of COX-2 in the presence or absence of H_2S may therefore provide clearer depiction of NF-κB regulation by H_2S. However, COX-2 may

be regulated by other pathways independent of NF-κB. Indeed, MAPK pathways have been shown to regulate COX-2 stability at the transcriptional and post-transcriptional level (Tsatsanis, et al. 2006). Furthermore, transcription factor CCAAT/enhancer-binding protein (C/EBP) has also been implicated in COX-2 regulation in endothelial cells (Kang, et al. 2007).

INOS is also another downstream affector of the NF-κB pathway (Xie, et al. 1994). Due to technical difficulties in measuring iNOS protein expression in HUVEC, an alternative approach to examinine the effects of H_2S on iNOS expression was needed. The measurement of nitrate/nitrite production from LPS stimulated RAW264.7 cells is a robust and relatively quick method to examine whether GYY4137 effects iNOS activity and/or expression. Both GYY4137 and NaHS could concentration dependently reduce nitrate/nitrite production from LPS stimulated RAW264.7. This correlates with another study that deomonstrated that GYY4137 and NaHS could inhibit nitrate/nitrite production from LPS stimulated RAW264.7 cells (Whiteman, et al. 2010c). Interestingly, NaHS has been shown to inhibit LPS induced iNOS expression in RAW264.7 (Oh, et al. 2006) and microglia (Hu, et al. 2007) which may suggest H_2S is capable of inhibiting NF-κB translocation to the nucleus. However, like COX-2, iNOS is also regulated by other transcription factors such as STAT (Bolli, et al. 2001) and AP-1 (Cho, et al. 2002).

E-selectin has also been reported to be regulated by NF-κB (Ehrhardt, et al. 2004). However, in the current study, H_2S did not inhibit soluble E-selectin expression (Figure 5.2.2). If H_2S was to inhibit NF-κB translocation to the nucleus H_2S should also inhibit E-selectin expression which was not the case in the current study. E-selectin is maximally expressed at 4 h and rapidly declines at 24 h (Ghersa, et al. 1997). In

contrast, VCAM-1 expression has been reported to be maximal at 6-8 h and unlike E-selectin its expression is relatively stable at 24 h (Ghersa, et al. 1997). The different kinetics in the regulating adhesion molecules suggests alternative mechanisms of adhesion molecule regulation which may be independent of NF-κB. In a previous study, shear stress was shown to increase both protein and mRNA levels of TNF-α induced ICAM-1, but reduced TNF-α induced VCAM-1 and E-selectin in HUVEC (Chiu, et al. 2004), demonstrating different regulatory mechanisms between each of the adhesion molecules that may not be solely related to the NF-κB translocation pathway.

H_2S itself has also been demonstrated to act on a number of different signalling pathways. For example, H_2S regulates the MAPK pathway. In HUVEC treated with TNF-α, p38 MAPK activation was inhibited by NaHS (100 μM, pre-treatment) but had no effect on ERK1/2 or JNK1/2 (Pan, et al. 2011). In another study, HUVEC exposed to H_2S (60 μM) induced a sustained phosphorylation of ERK1/2 and a transient phosphorylation of p38 MAPK (Papapetropoulos, et al. 2009). Indeed, a number of MAPK pathways are also capable of integrating with NF-κB pathway as demonstrated in a number of tissues: human kidney specimens (Sakai, et al. 2002), human bronchial epithelial and eosinophils (Wong, et al. 2005), bovine chondrocytes (Chowdhury, et al. 2008) and murine embryonic fibroblasts (Tang, et al. 2001, Papa, et al. 2006).

The activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway is involved in cell cycle progression (Liang and Slingerland 2003), the regulation of protein trafficking (Wurmser, et al. 1999), and the regulation of angiogensis (Cai, et al. 2007). H_2S has been shown to activate this pathway in HUVEC (Papapetropoulos, et al. 2009) and RF/6A endothelial cells (Cai, et al. 2007). Interestingly, the activation of PI3K/AKT pathway has been shown to negatively

regulate the NF-κB pathway in a model of acute pancretitis induced by caerulein (Tamizhselvi, et al. 2009).

Other pathways of H_2S activation may include antioxidant pathways. TNF-α stimulated HUVEC exposed to NaHS (10-100 μM) has been shown to increase the protein expression of HO-1 (Pan, et al. 2011). This may suggest that H_2S is capable of regulating the nuclear factor erythroid 2-related factor (Nrf2) antioxdant pathway in endothelial cells. Interestingly, this antioxidant pathway has also been shown to negatively regulate the NF-κB pathway in a mouse model of traumatic brain injury (Jin, et al. 2008).

Overall, H_2S affects a number of different signalling pathways and it is unlikely there is a single straight forward pathway for the regulation of adhesion molecule expression. Indeed, it is possible that H_2S may indirectly inhibit NF-κB translocation through affecting other signalling pathways such as MAPK, PI3K/AKT or Nrf2. The integration of several signalling pathways at one time is probable and likely to be dependent on the stimuli, cell type, timing, concentration of H_2S and cell environment.

In the current study, GYY4137 and NaHS significantly reduced ICAM-1 and VCAM-1 expression but not E-selectin expression. This effect may be partially mediated by regulating the NF-κB pathway, however further study is necessary to confirm this. Indeed, alternative methods to measure NF-κB activation include the measurement of IκB-α phosphorylation, NF-κB nuclear translocation and NF-κB DNA binding. The reason for the selectivity of H_2S on ICAM-1 and VCAM-1 expression is unknown but may be due a number of variables discussed above.

5.3.3. GYY4137 and oedema induced by carrageenan in the mouse hindpaw

To determine whether GYY4137 has an effect on MPO activity, pain and oedema formation *in vivo*, the carrageenan hindpaw model was used. In the current study, GYY4137 (50 mg/kg i.p. 1 h post carrageenan) had no significant effect on paw oedema (Figure 5.2.19). In contrast to the current work, another study demonstrated that injection of NaHS (150 µmol/kg i.p., 30 min pre-treatment) significantly reduced carrageenan induced paw oedema in rats (Zanardo, et al. 2006). Similarly, others have demonstrated that S-diclofenac (H_2S releasing NSAID) was more potent than diclofenac at reducing carrageenan induced paw oedema in rats (Sidhapuriwala, et al. 2007). The reason behind the difference in the current study and the two latter studies, may be due to the use of different H_2S donors (GYY4137 c.f. NaHS/s-diclofenac), species (mouse c.f. rat), timing (post-treatment c.f. pre-treatment) and/or different methods to measure oedema (paw weight/size c.f. paw volume using a plethysmometer).

In contrast to administrating H_2S donors (Zanardo, et al. 2006, Sidhapuriwala, et al. 2007), one study demonstrated that PAG (25 – 75 mg/kg i.p., 60 min pre-treatment) dose dependently reduced carrageenan-induced hindpaw oedema in rat (Bhatia, et al. 2005), suggesting that endogenous and exogenous H_2S exert different effects. Indeed, the controversies on oedema formation with vasodilators have also been demonstrated with $^{\bullet}NO$. $^{\bullet}NO$ donors have been shown to reduce oedema formation in the mouse carrageenan hindpaw model (Fernandes, et al. 2002). In contrast, administration of L-NAME (NOS inhibitor, 50 mg/kg) has also been shown to reduce oedema formation in the rat carrageenan hindpaw model (Lazzarini, et al. 2006).

Indeed, it is difficult to predict whether a vasodilator like H_2S may reduce or enhance oedema peripherally. Increasing blood flow should in theory increase perfusion pressure and enhance oedema formation. However, both $^{\bullet}NO$ (Fernandes, et al. 2002) and H_2S donors (Zanardo, et al. 2006) have been demonstrated to reduce oedema formation. However, the anti-oedema effects induced by these agents may not be a direct effect as a result of their vasoactive properties. There are several mediators involved in increasing vascular permeability, namely serotonine (5-hydroxytryptamine 5-HT, released from mast cells, platelets and noradrenergic nerve terminals), histamine (predominately released from mast cells) and prostaglandins (synthesised by COX-1 and COX-2, both ubiquitously expressed) (Posadas, et al. 2004). H_2S donors have been demonstrated to inhibit COX-2 expression (Hu, et al. 2008a, Yang, et al. 2011a) and prostaglandin production (Whiteman, et al. 2010c). In rats, COX-2 message and PGE_2 is up-regulated in carrageenan hindpaws within 1 h of injection (Nantel, et al. 1999). Three hours after carrageenan injection, immunohistochemical analysis suggests that COX-2 is predominately expressed in the stratum corneum of the epidermis, inflammatory cells and depending on the severity of the inflammation, skeletal muscle cells (Nantel, et al. 1999). The inhibition of COX-2 expression may be a reason for the H_2S induced reduction in carrageenan-induced paw oedema described in previous studies (Zanardo, et al. 2006). Whether GYY4137 inhibits COX-2 expression and prostaglandin synthesis, or alternative mediators involved in vascular permeability (e.g. histamine or 5-HT), in the carrageenan model of inflammation requires further study.

In mice, the carrageenan induced paw-oedema model is biphasic, with the first phase of swelling occurring between 1-6 h and the second phase of further swelling

occurring between 24 – 96 h (Posadas, et al. 2004). In CD-1 mice, the expression of COX-2 protein induced by carrageenan occurs at later time points (>6 h), typically in the second phase of carrageenan induced paw oedema (Posadas, et al. 2004). In the current study, the carrageenan paw-oedema model was terminated at 6 h due to home office licence requirements. Indeed, it would be interesting to determine whether GYY4137 would have more prominent effects on paw oedema in the second phase of this carrageenan model as GYY4137 has been demonstrated to inhibit COX-2 expression at 24 h in HUVEC (Figure 5.2.13).

5.3.4. GYY4137 and hyperalgesia in the carrageenan paw-oedema mouse model

In the current study, GYY4137 did not have an effect on mechanical hyperalgesia (Figure 5.2.20). In an inflammatory model of arthritis (2% w/v kaolin- 2% w/v carrageenan), an intra-articular injection of Na_2S (60 µM in 100 µl, 24 h post-insult) significantly ($P<0.001$) reduced leukocyte adhesion (Andruski, et al. 2008). However in the same model of inflammation, Na_2S (100 µM in 100 µl) had no effect on joint pain measured using von Frey hair algesiometry (Andruski, et al. 2008), which accords with the current study. However, in contrast another study utilising the carrageenan-induced knee joint synovitis model, pre-treatment with the H_2S donor Lawesson's reagent (3.6 µmol per joint, 60 min) ameliorated the severity of knee joint pain, as measured by a behavioural observation gait score (Ekundi-Valentim, et al. 2010).

The role of H_2S in pain is still unclear. Both pro-nociceptive (co-injection of NaHS 1 nmol/0.1 mL and formalin 1.25% in rat hindpaw (Lee, et al. 2008), NaHS 0.5 – 5 nmol/mouse intracolonic (Matsunami, et al. 2009), L-cysteine 100 nmol/paw i.pl. in rat

(Kawabata, et al. 2007), CBS inhibitor hydroxylamine 25 µmol/kg in rat colorectal distension) and anti-nociceptive (NaHS 30 µmol/kg i.p. in a rat model of colorectal distension (Distrutti, et al. 2006a), Lawesson's reagent 3.6 µmol per joint in a carrageenan knee joint model (Ekundi-Valentim, et al. 2010)) effects of H_2S have been described. These nociceptive effects have been suggested to involve T-type calcium channels (Kawabata, et al. 2007, Matsunami, et al. 2009), K_{ATP} channels (Distrutti, et al. 2006a) and transient receptor potential A1 (TRPA1) ion channels (Streng, et al. 2008).

In the current study, GYY4137 post-treatment had no effect on reducing mechanical nociceptive threshold. It would be interesting to determine whether GYY4137 pre-treatment would have an effect on mechanical hyperalgesia and whether other pain thresholds such as thermal nociceptive threshold would be affected.

5.3.5. GYY4137 and MPO activity in carrageenan hindpaws

Although GYY4137 did not significantly reduce paw oedema or hyperlagesia in the current study, GYY4137 significantly ($P<0.05$) reduced MPO activity, an indicator of neutrophil infiltration, in carrageenan hindpaw homogenates compared to control (Figure 5.2.21) implying perhaps an effect on leukocyte trafficking which may be secondary to inhibition of adhesion molecule expression reported in this chapter. Indeed, it has previously been demonstrated that H_2S can inhibit leukocyte infiltration in carrageenan models of inflammation: pre-treatment with NaHS (100 µmol/kg i.p.), 30 min prior to carrageenan injection into the air pouch of rats, significantly ($P<0.05$) reduced the leukocyte exudates cell count compared to control (Zanardo, et al. 2006); 3 h pre-treatment with S-diclofenac (EC_{50} 12.0 ± 2.1 µmol/kg, i.p.) was more potent than diclofenac (EC_{50} 21.9 ± 2.0 µmol/kg i.p.) at inhibiting MPO activity in the

carrageenan hindpaws of rats (Sidhapuriwala, et al. 2007); Lawesson's reagent (H_2S donor, 3.6 μmol per joint, 60 min pre-treatment) was shown to reduce total, neutrophil and lymphocyte cell count in the synovial lavage fluid from rats with carrageenan-induced synovitis (Ekundi-Valentim, et al. 2010).

Interestingly, the incubation of GYY4137 (5 μM) with purified MPO *in vitro* significantly reduced MPO enzyme activity (Figure 5.2.22) suggesting the reduction of MPO activity in carrageenan hindpaws induced by GYY4137 may not be as a consequence of leukocyte infiltration but due to the inhibition of MPO enzyme activity itself. Interestingly, another study using purified MPO, demonstrated that NaHS (35 – 500 μM) could concentration dependently reduce MPO activity (Laggner, et al. 2007). Indeed, further work is necessary to determine whether GYY4137 directly reduces leukocyte infiltration, possibly by cell count or histochemical analysis. Moreover, an *in vitro* assay to measure leukocyte adherence to stimulated HUVEC in the presence or absence of H_2S will provide further evidence for a functional effect of reducing VCAM-1/ICAM-1 *in vitro*. Interestingly, in a rat model of endotoxin shock, GYY4137 did not reduce MPO activity in liver or lung when injected 1 h prior to LPS insult (Li, et al. 2009b). This implies that, although GYY4137 can directly inhibit MPO activity *in vitro* (Figure 5.2.22), GYY4137 does not directly affect MPO enzyme activity *in vivo*.

In correlation with the overall anti-inflammatory effect of GYY4137 in this study, others have shown that GYY4137 (50 mg/kg i.p.) injected 1 h post-treatment (but not 1 h pre-treatment) significantly reduced MPO activity in the lung of septic rats (LPS 4 mg/kg i.p), suggesting GYY4137 inhibits leukocyte infiltration (Li, et al. 2009b). Moreover, GYY4137 reduced the severity of tissue damage in the lung and liver induced by LPS, confirmed by histology (Li, et al. 2009b). Furthermore, these authors

demonstrated a reduction in plasma nitrate, L-selectin, TNF-α, IL-1β and IL-6 on injection of GYY4137 1 h post insult (Li, et al. 2009b). In addition, the administration of decomposed GYY4137 did not significantly alter plasma TNF-α or tissue MPO activity suggesting that the H_2S released from GYY4137 is the reason for the anti-inflammatory effects demonstrated in this model of inflammation (Li, et al. 2009b). In another study GYY4137 was shown to inhibit IL-1β, TNF-α, PGE_2 and nitrite in LPS stimulated RAW264.7 macrophages (Whiteman, et al. 2010c). Moreover, these effects were shown to be through inhibiting NF-κB activation (NF-κB DNA binding), HSP-27 (heat-shock-protein-27) phosphorylation and ATF2 (activating transcription factor 2) phosphorylation (Whiteman, et al. 2010c). Moreover, GYY4137 also been demonstrated to exhibit antioxidant properties, as demonstrated by its ability to inhibit apoptosis induced by H_2O_2 in human chondrocytes (Fox, et al. 2011).

5.1.1. Conclusion

The current study has demonstrated that H_2S donors GYY4137 and NaHS are capable of reducing VCAM-1 and ICAM-1, but not E-selectin expression on HUVEC. In contrast to NaHS the effects of GYY4137 on HUVEC are unlikely to be a direct effect on cell toxicity. The inhibitory effects of GYY4137 on adhesion molecule expression on HUVEC correlate with a reduction in MPO activity in carrageenan hindpaws. In the current study, GYY4137 presents an overall anti-inflammatory effect. However, the effects of GYY4137 on MPO activity may not be a direct effect on leukocyte infiltration and further study is required to confirm this. The slow release of H_2S from GYY4137 is the foremost beneficial advantage of this H_2S donor compared to the conventional H_2S-salts and is likely to be the cause of the anti-inflammatory effects described in this study and is a useful tool to further our understanding in the H_2S field.

CHAPTER 6: Investigation and Characterisation of Novel Antioxidant ZJ802

6. Introduction

NaHS has been demonstrated to have both pro- and anti- inflammatory effects in different models of inflammation. Many of the anti-inflammatory properties of H_2S donors may be in part due to their ability to act as antioxidants. Indeed, H_2S donors have also been demonstrated to scavenge reactive oxygen species such as H_2O_2, HOCl (Laggner, et al. 2007), $ONOO^-$ (Whiteman, et al. 2004), $O_2^{\bullet-}$ (Chang, et al. 2008) and $^{\bullet}NO$ (Whiteman, et al. 2006, Fox, et al. 2011). However, the pro- inflammatory and pro- apoptotic effect of H_2S donors may arise as a result of the nature of the H_2S donor. Indeed, some of the problems with the traditional H_2S donors, NaHS or Na_2S, is that H_2S is released almost immediately in solution. Potential problems include the release of high instantaneous concentrations of H_2S which may be toxic to cells (Beauchamp, et al. 1984). GYY4137 a novel organic slow releasing H_2S donor has recently been shown to exert anti-inflammatory affects *in vivo* (Li, et al. 2009b) and *in vitro* (Whiteman, et al. 2010c), and data shown in Chapter 5 of this thesis supports an anti-inflammatory effect of GYY4137. In addition, GYY4137 has been shown to induce cell death selectively in tumour cell lines, but not non-cancer cell lines, suggesting that GYY4137 may have therapeutic potential in the treatment of cancer (Lee, et al. 2011). As mentioned in Chapter 5, there is a need for more organic H_2S donors in order to enhance our knowledge about the biological function of H_2S.

The aim of this chapter was:

1. Using biochemical techniques, characterise a library of potential H_2S releasing donors:
    a) For their ability to release H_2S, using the methylene blue, ISE and DTNB methods.
    b) For their antioxidant ability by assessing a) the ability to scavenge free radicals (ABTS and DPPH) and b) reducing capacity (ferricyanide reducing power) of these compounds.
2. On identifying a potent antioxidant, ZJ802, this compound was further characterised for potential anti-inflammatory actions *in vivo:* by characterising biochemical endpoints (MPO and plasma TNF-α and IL-6) in a mouse model of endotoxic shock.

6.1 Materials and Methods

6.1.1 ZJ802

ZJ802 was synthesised by Professor Tan Choon Hong (National University of Singapore, Singapore) m.w. 246.44.

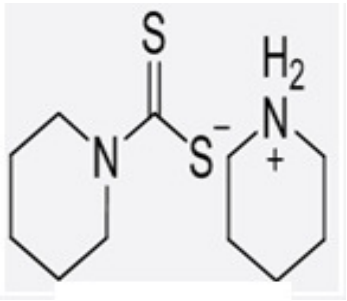

Figure 6.1.1. Structure of ZJ802

6.1.2 Animal housing

CD-1 male mice (Charles River, 25 g) were housed in King's College London Biological Services Unit (Waterloo) in a climatically controlled environment (45% humidity, 20^{o}C, 12 h light cycle). Laboratory chow and drinking water were provided *ad libitum*. All experiments were carried out according to the 1986 UK Home Office Animals Scientific Procedures Act.

6.1.3 Blood pressure measurement in anaesthetised mice

C57Bl6 (male, 25 g) were anesthetised under isoflurane (2.5% isofluorane induction, then 1.5% for maintenance using an air pump) placed on a homeothermic heating blanket (Harvard) in a supine position. Pressure catheter implantation was performed with the aid of a Leica M60 Stereo Zoom Microscope and Schott KL1500 LCD (220-240 V / 50/60 Hz) lighting system.

The skin of the throat was cut from the chin to the sternum and the right and left sternohyoid muscles were separated via blunt dissection exposing the trachea. The right carotid artery was subsequently located, cleaned of nerves and connective tissue, and three pieces of silk sulture (5.0) were placed under the vessel. A ligature was made to the distal part of the carotid artery to stop blood flow to the brain. A loose ligature was made using a single loose knot to temporarily occlude blood flow from the heart to prevent blood loss during cannulation of the vessel. A pair of hemostats were used to put tension on the artery to allow catheterisation using the suture ties. A modified 23G needle (tip bent 90^{o}) was used to carefully puncture a hole in the carotid artery between the two ligatures and guide the catheter (1.2F Pressure Catheter, Scisense) into the vessel. Once in the correct position to record arterial pressure the catheter was secured in the vessel with a final suture. Pressure calibration, signal transduction and amplification were made by a FP891B pressure control unit (Scisense). Data was acquired using a Powerlab (35 series) and LabChart software (ADInstruments). The jugular vein was exposed for intravenous delivery of drugs. Following 10 min of stable arterial blood pressure recording an insulin needle (30G) was used to deliver LD802 (203 µmol/kg), GYY4137 (133 µmol/kg), NaHS (18 µmol/kg) (Li, et al. 2008b) or vehicle (saline, 2.5 ml/kg). Blood pressure was subsequently monitored for up to 1 h.

6.1.4 LPS and ZJ802 administration

ZJ802 (50 mg/kg, 10 ml/kg i.p., 27 G needle) or saline (10 ml/kg i.p. , 0.9% w/v sodium chloride) was injected into conscious male CD-1 mice 15 min prior to or 30 min post-LPS injection (Escherichia coli, serotype O127:B8; 10 mg/kg, 10 ml/kg i.p.). Mice were immediately killed via cervical dislocation after whole blood was obtained in

heparinized syringes (50,000 units/ml) via cardiac puncher in anaesthetised mice (isoflourane 4% v/v), 4 h after LPS administration. Whole blood was centrifuged (2000 g, 20 min) and the plasma collected. Organs (heart, lung, liver, kidney and spleen) were subsequently collected, rinsed with saline, snap-frozen in liquid nitrogen and stored at -80^{o}C for assay later.

6.1.5 H_2S release from ZJ802 using the ISE and methylene blue assay

ZJ802 was dissolved in phosphate buffer (100 mM, $KHPO_4$ pH 7.5) and the release of H_2S from ZJ802 was measured by the methylene blue colourimetric assay (Method 2.9) or the ISE (Method 2.10).

6.1.6 H_2S release from ZJ802 using DTNB

Due to inconsistent results obtained between the methylene blue colourimetric assay and ISE, the DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) assay was used as an additional method used to measure H_2S release from ZJ802.

DTNB, also known as Ellmans reagent, is used to measure thiol (-SH) groups (Ellman 1959) and has been previously used to measure glutathione production and sulfhydryl groups bound to proteins (Sedlak and Lindsay 1968). DTNB is reduced by thiol groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH (Sedlak and Lindsay 1968). The nitromercaptobenzoic acid anion, has an intense yellow colour that is most stable at pH 8.0, which is detected spectrophotometrically (Sedlak and Lindsay 1968). Due to its non-specific properties to measure thiols, this assay was only used to measure H_2S in protein free *in vitro* conditions.

To determine whether ZJ802 was an H_2S releasing agent ZJ802 (5 mM) was incubated in water, $KHPO_4$ (100 mM, pH 4.5) or $KHPO_4$ (100 mM, pH 7.5) at room

temperature or 37^{o}C for the indicated periods of time. A standard curve using NaHS (4 – 500 µM) was used as an indicator of H_2S release. H_2S (20 µl) was added to 96 well plates containing DTNB (50 µl, 1 mM in water) and HEPEs buffer (50 µl, 1 M pH 8.0). After 10 min incubation at room temperature and the absorbance measured at 421 nm.

6.1.7 MPO activity in mouse tissues

Tissues from animals were snap frozen in liquid nitrogen within 5 min of death and stored at -80^{o}C until use. Liver, lung and kidney tissues (5% w/v) were homogenised in ice-cold buffer 1 (20 mM $KHPO_4$, pH 7.4) using a Polytron Homogeniser. Homogenates were subsequent centrifuged at 13,000 g for 10 min at 4^{o}C. The supernatant was discarded and the pellet resuspended in 1 ml buffer 2 (50 mM $KHPO_4$, 0.5% w/v hexadecyltrimethylammonium bromide (HTAB), pH 6). The suspension was subsequently snap frozen and thawed at 37^{o}C, 4 times. The suspension was then sonicated for 10 sec at 20 amps followed by 20 sec rest in ice cold water, 4 times. Samples were then centrifuged at 13,000 g for 5 min. Supernatant (50 µl) was added into each well of a 96-well plate followed by TMB/H_2O_2 substrate (100 µl) and incubated at room temperature for 30 min. The absorbance was subsequently read at 652 nm. A standard curve for MPO was constructed from purified MPO derived from human leukocytes (0.1 – 1 units MPO/ml, Sigma M6908). Values were standardised to protein, using Bradford protein assay (Method 2.6). Data was subsequently expressed as MPO units/mg protein.

6.1.8 Plasma TNFa and IL-1

TNF-α and IL-6 levels in mouse plasma were determined via an Enzyme-Linked Immunosorbent Assay (ELISA), ab46105 and ab46100 (Abcam) respectively. Plasma was diluted 1:400 in the standard diluent and the assay conducted according to the manufacturers instructions.

6.1.9 ABTS antioxidant assay

ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid, 7 mM) was converted to its free radical form ($ABTS^{`+}$) through incubation with the oxidising agent potassium persulfate ($K_2S_2O_8$, 2.5 mM) for a minimum of 12 h in the dark (Re, et al. 1999). In the presence of $H^+$ donating antioxidants $ABTS^{`+}$ is reduced and decolourises. The $ABTS^{`+}$ was diluted using PBS to an absorbance of 0.6 at 734 nm. $ABTS^{`+}$ solution (1 ml) was added to varying concentrations of ZJ802 (10 µl, 30-1000 µM, PBS) and incubated at room temperature for 5 min before the absorbance read at 734 nm. Results were subsequently plotted as the absorbance reading at 734 nm.

6.1.10 DPPH antioxidant assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a free radical (Ates, et al. 2008). This assay is based in the ability of antioxidants to reduce DPPH to DPPHH. A reduction in DPPH colour intensity indicates increased radical scavenging power. Varying concentrations of ZJ802 (50 µl, 30-1000 µM, H_2O) was added to DPPH (1.45 ml, 10 mM, methanol) and left in the dark for 30 min at room temperature before being measured at 520 nm. Results were subsequently plotted as % control of the absorbance reading at 520 nm.

6.1.11 Ferricyanide reducing power

This assay measures the reducing power of a compound, an indicator of antioxidant capacity (Ates, et al. 2008). In brief, potassium ferricyanide ($K_3[Fe(CN)_6]$, 250 µl, 1% w/v in H_2O) and $KHPO_4$ (250 µl, 0.2 M, pH 6.6) was added to ZJ802 (100 µl, 30 – 1000 µM, H_2O) and incubated at 50^{o}C for 20 min. During this reaction, the ferricyanide ion is reduced to ferrocyanide ($[Fe(CN)_6]^{4-}$) in the presence of reducing agents (Lillie and Donaldson 1974). The reaction was terminated by addition of TCA (250 µl, 10% w/v in H_2O) and centrifuged at 13,000 g for 5 min. 250 µl of this solution was added to a tube containing 250 µl H_2O and 50 µl ferric chloride ($FeCl_3.6H_2O$, 0.1% w/v H_2O). Here, ferric ions react with ferrocyanide to form a blue colour, Prussian Blue (Lillie and Donaldson 1974). After incubation for 5 min at room temperature, the absorbance was read at 700 nm. Results were subsequently plotted as the absorbance reading at 700 nm.

6.2 Results

6.2.1 H_2S release from ZJ802

H_2S release from ZJ802 (1 mM, pH 7.4, n = 4, time = 0) was 11.07 ± 1.31 µM H_2S according to the ISE (Figure 6.2.1). However, H_2S was undetectable using the methylene blue assay. As a result the DTNB assay, which measures the presence of thiol (-SH) groups, was utilised as an alternative indicator of H_2S release. The DTNB assay had a sensitivity from 15 µM H_2S and was linear up to 10 mM H_2S (Figure 6.2.2). Based on the results obtained from the DTNB assay, ZJ802 resulted in a dose dependent rise in -SH concentrations (Figure 6.2.3) an effect that was pH and temperature dependent (Figure 6.2.4).

The release of -SH groups from GYY4137 is also pH and temperature dependent (Figure 6.2.4) as previously described in the literature (Li, et al. 2009b). The -SH release profile of ZJ802 was similar to that of NaHS (previously described in Chapter 5), that is the release of -SH groups was immediate and gradually declined over time (Figure 6.2.4). This is in contrast to the slow releasing donor GYY4137 which released –SH groups gradually over time, leading to the steady accumulation of H_2S/-SH groups in solution (Figure 6.2.4).

6.2.2 ZJ802 and blood pressure in anesthetised mice

As H_2S donors have been shown to reduce blood pressure (BP) (Zhao, et al. 2001). As a result, studies were performed to investigate the blood pressure effects of ZJ802. A bolus injection of ZJ802 (50 mg/kg i.v.) was administered into anesthetised mice. ZJ802 caused an almost immediate drop in blood pressure (mean BP from 93.40 ± 1.50 to 50.57 ± 8.11 mmHg, n = 3) which increased over 30 min but did not reach original baseline values over the time course studied (Figure 6.2.5 and 6.2.6).

NaHS was injected into the mice after injection of ZJ802, as a positive control and to compare the antihypertensive kinetics of both donors. The blood pressure induced by NaHS (1 mg/kg i.v.), caused an immediate transient drop in blood pressure (mean BP from 79.10 ± 22.31 c.f. 55.24 ± 33.57, n = 2) which lasted approximately 30 sec before reaching baseline (Figure 6.2.7) as previously reported (Zhao, et al. 2001).

To ensure the blood pressure effect of ZJ802 was not a result from altering endogenous pH levels, the pH of this compound was examined. ZJ802 had a pH of 7.15 in saline and hence it is unlikely that the observed blood pressure effects occurred as a result of rapid changes in pH following drug administration.

6.2.3 Antioxidant capacity of ZJ802

H_2S donors have antioxidant properties and have previously been shown to scavenge radicals such as peroxynitrite ($ONOO^-$) (Whiteman, et al. 2004) and superoxide ($O_2^{\bullet-}$) (Chang, et al. 2008). The antioxidant and reducing capacity of H_2S donors (NaHS and GYY4137) were compared to ZJ802. All compounds dose dependently reduced ferricyanide to ferrocyanide (Figure 6.2.8 a). ZJ802 was a more potent reducing agent than both NaHS and GYY4137 (Figure 6.2.8 a).

Two free radical scavenging assays were used to determine the antioxidant capacity of NaHS, GYY4137 and ZJ802. All compounds dose dependently scavenged free radicals generated by DPHH (Figure 6.2.8 b) and ABTS (Figure 6.2.8 c). ZJ802 was comparable to NaHS at scavenging DPHH (Figure 6.2.8 b) and more potent than both NaHS and GYY4137 at scavenging ABTS (Figure 6.2.8 c).

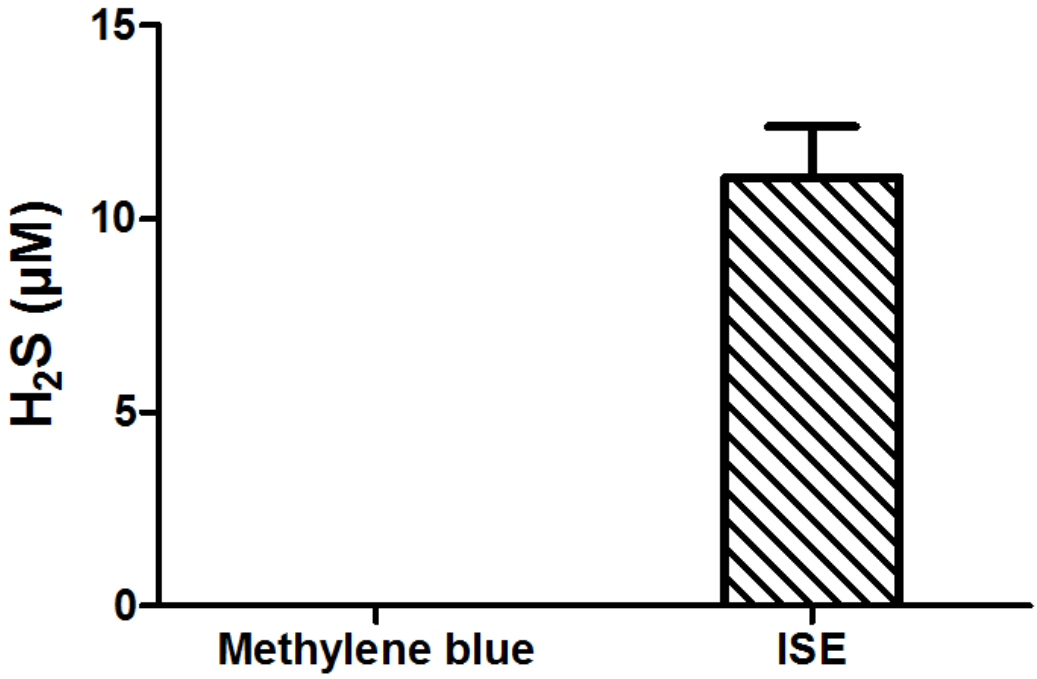

Figure 6.2.1. H_2S release from ZJ802 (1 mM) in $KHPO_4$ (100 mM, pH 7.4) measured using the methylene blue assay, and ISE. H_2S was immediately measured once solubilising ZJ802. Data are shown as H_2S µM as mean ± SEM, n = 4.

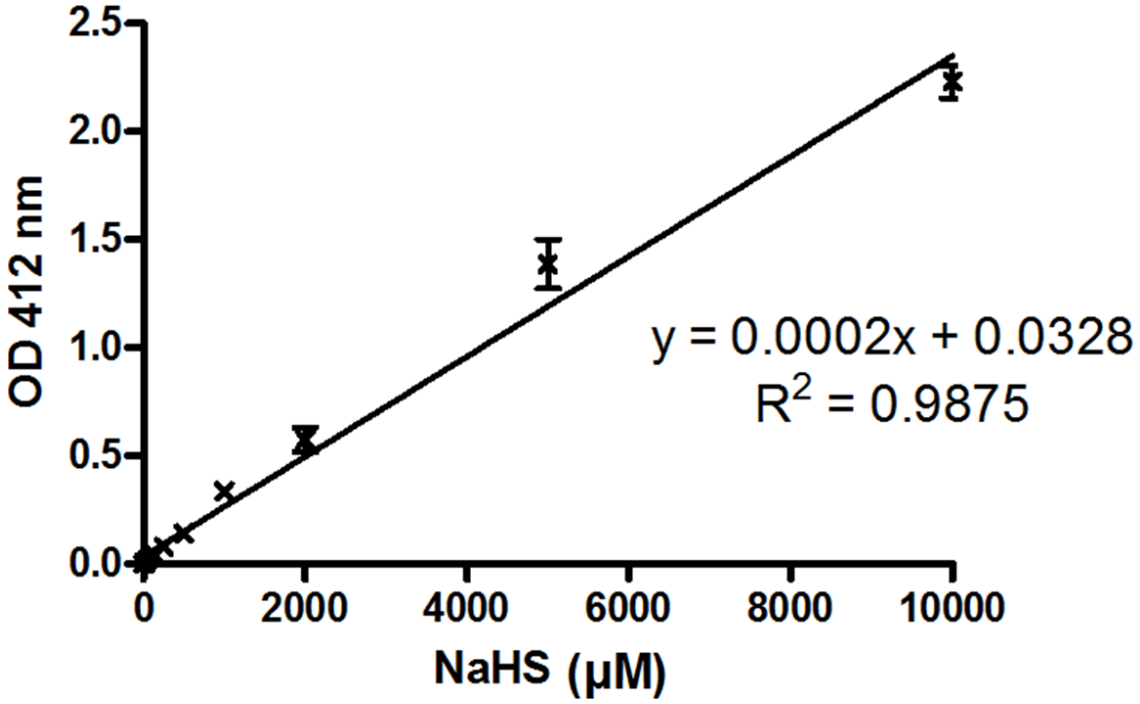

Figure 6.2.2. DTNB standard curve using NaHS (4 – 10000 µM). Data are shown as mean ± SEM, n = 3.

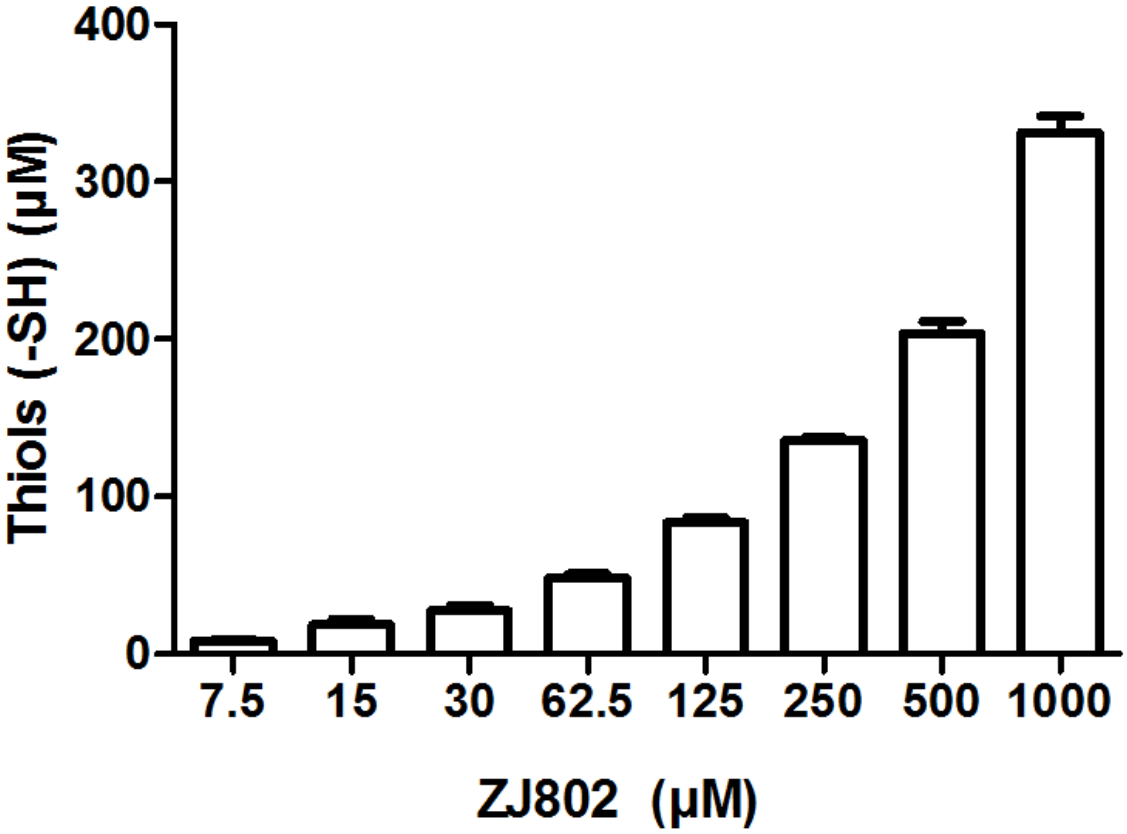

Figure 6.2.3 Increasing concentration of thiol (-SH) groups with increasing concentrations of ZJ802 (7.5 – 1000 µM) in $KHPO_4$ (100 mM, pH 7.4) as measured by DTNB assay. Data show thiols (µM) as mean ± SEM, n = 3.

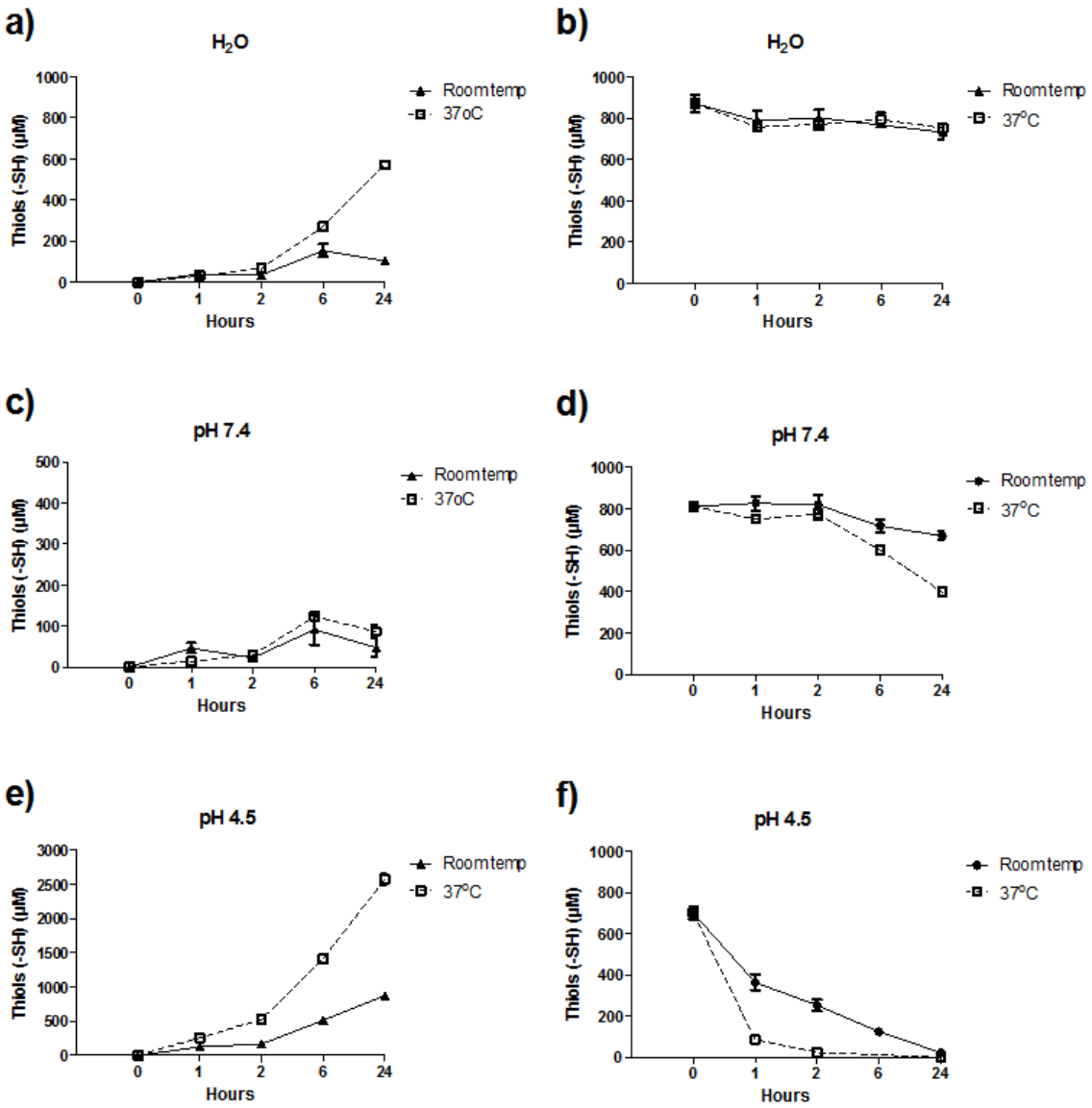

Figure 6.2.4. GYY4137 (5 mM) was dissolved in a) H_2O c) $KHPO_4$ (100 mM pH 7.4) e) $KHPO_4$ (100 mM pH 4.5). ZJ802 (5 mM) was dissolved in b) H_2O d) $KHPO_4$ (100 mM pH 7.4) f) $KHPO_4$ (100 mM pH 4.5) and incubated at room temperature or 37°C over the indicated time. Data are shown as mean ± SEM, thiol (-SH) groups (µM) determined by the DTNB assay, n = 3.

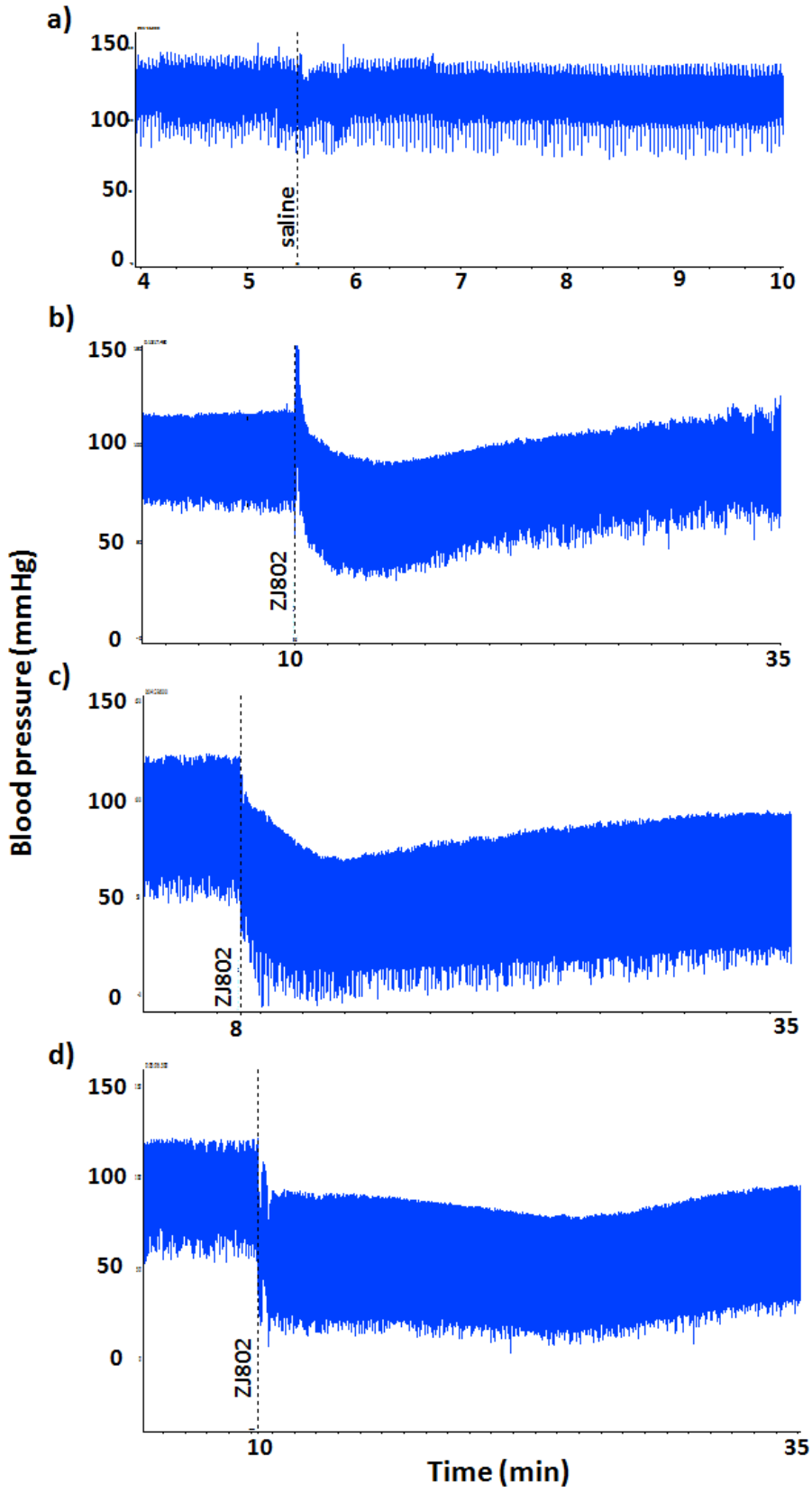

Figure 6.2.5. Blood pressure traces using anaesthetised mice. Mice were injected with a) saline (2.5 ml/kg i.v.) or b) c) and d) ZJ802 (50 mg/kg, i.v.). Data are shown as individual blood pressure traces from 4 mice.

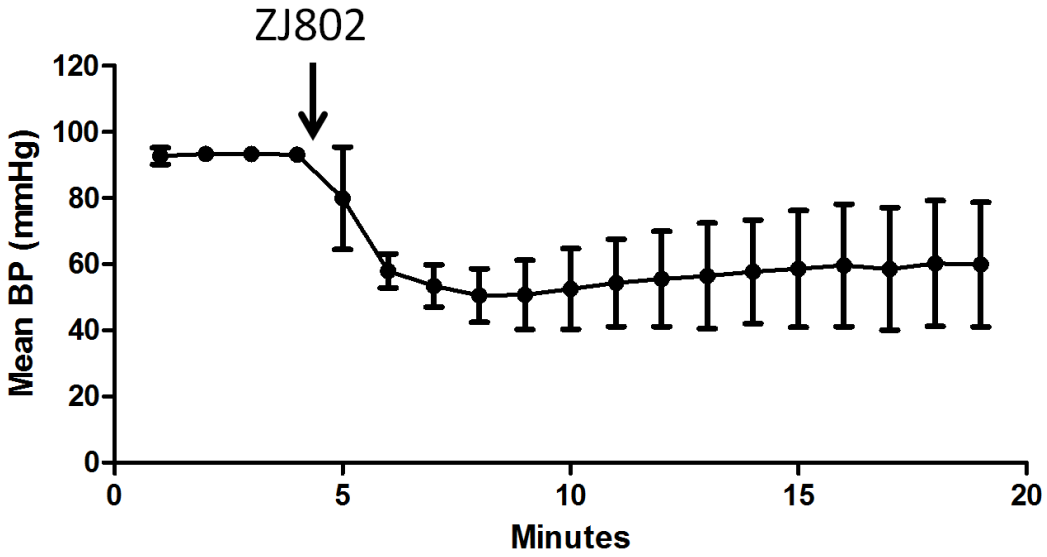

Figure 6.2.6. Mean blood pressure (BP) traces in anaesthetised mice. Mice were injected with ZJ802 (50 mg/kg, i.v.). Data show mean ± SEM, n = 3

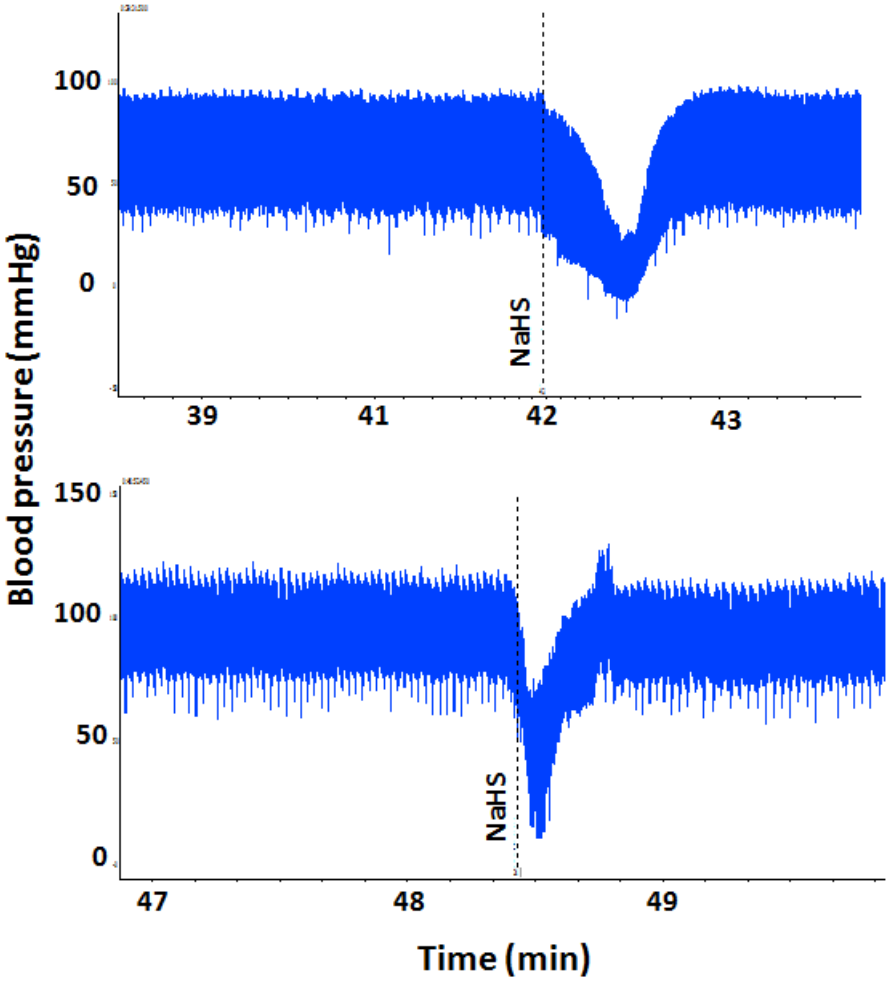

Figure 6.2.7. Blood pressure traces of anaesthetised mice injected with NaHS (1 mg/kg, i.v.) preconditioned with an injection of ZJ802 (50 mg/kg, i.v.). Data show individual blood pressure traces from 2 mice.

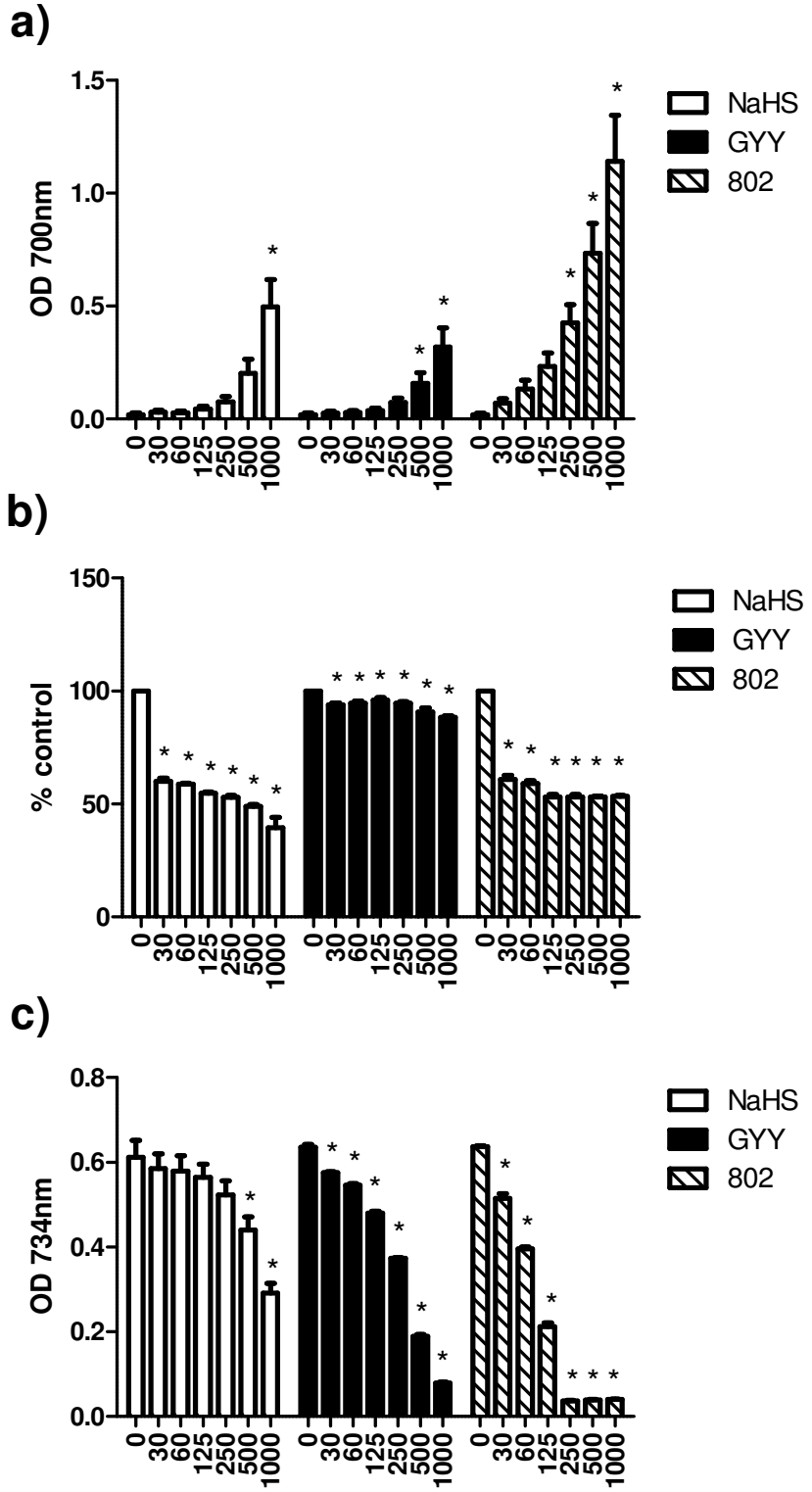

Figure 6.2.8. NaHS, GYY4137 or ZJ802 (30 – 1000 μM) were assayed for their antioxidant capacity as measured by a) ferricyanide reducing assay b) DPPH free radial scavenging assay c) ABTS free radial scavenging assay. Data show mean ± SEM, n = 5. *P<0.05 c.f. 0

6.2.4 ZJ802 in a mouse model of endotoxin shock

The effect of ZJ802 was examined further in an *in vivo* model of endotoxic shock. Mice were pre-treated (15 min) with ZJ802 (50 mg/kg, i.p.) prior to stimulation with LPS (10 mg/kg, i.p.). A significant ($P<0.05$) reduction in MPO activity, an indicator of leukocyte infiltration, was observed in lung (% inhibition 39.31 ± 9.04) and liver (% inhibition 49.16 ± 15.43) but not kidney (Figure 6.2.10 a, c and e). This anti-inflammatory effect of ZJ802 correlated with a significant reduction ($P<0.05$) in plasma IL-6 (% inhibition 50.57 ± 12.81) but not TNF-α (Figure 6.2.11).

However, ZJ802 (50 mg/kg, i.p.) injected 30 min post LPS did not show anti-inflammatory activity as demonstrated by an inability to affect MPO activity in lung, liver or kidney (Figure 6.2.10 b, d and f).

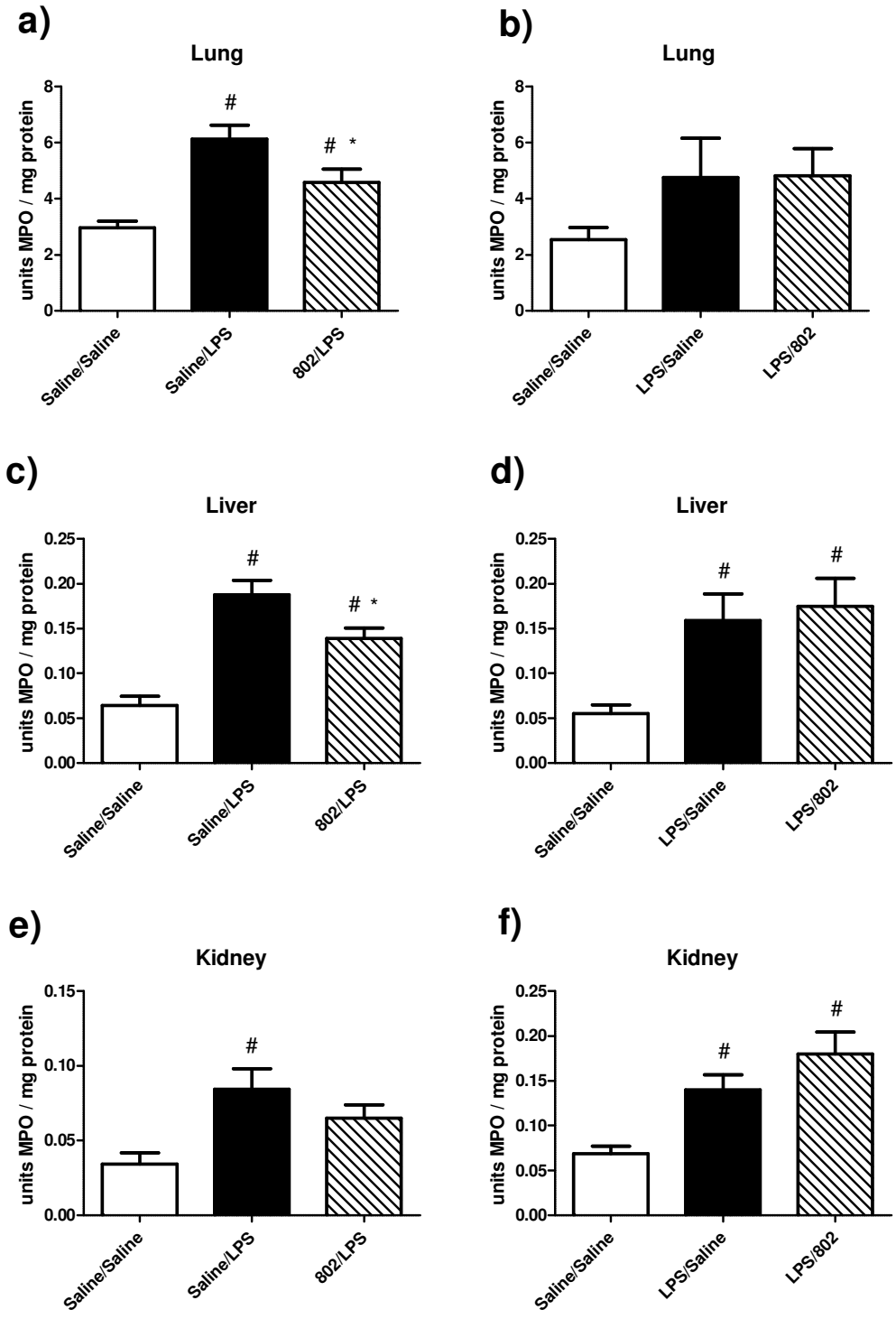

Figure 6.2.10. MPO activity in lung, liver and kidney of LPS (10 mg/kg i.p.) treated mice. Mice were pre-treated (15 min) with ZJ802 (50 mg/kg) and MPO was measured in a) lung c) liver e) kidney, 6 h post LPS injection. ZJ802 (50 mg/kg) was injected 30 min after LPS and MPO activity was measured in b) lung d) liver f) kidney, 6 h post LPS. Data shown as mean ± SEM, n = 6. #P<0.05 c.f. saline control, *P<0.05 c.f. LPS control.

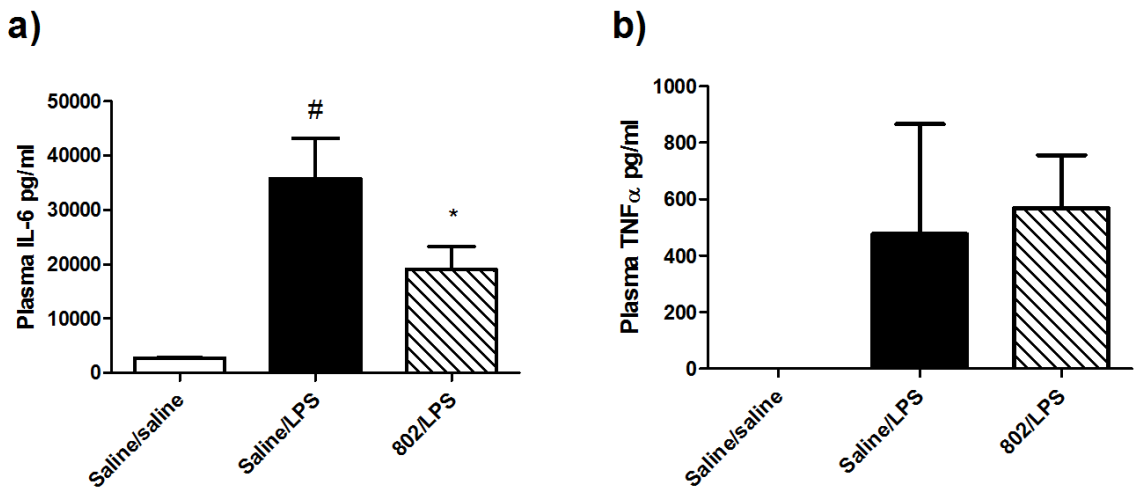

Figure 6.2.11. Mice were pre-treated with ZJ802 (50 mg/kg, i.p.) 15 min prior to LPS injection (10 mg/kg, i.p.). Plasma from mice was collected via cardiac puncher at 45 min or 4 h after LPS injection. Plasma levels of a) IL-6 (4 h LPS) or b) TNF-α (45 min LPS), were measured via ELISA. Data shown mean ± SEM, n = 4-5, #P<0.05 c.f. saline control, *P<0.05 c.f. LPS control

6.3 Discussion

The main findings from this chapter are:

1. ZJ802 is not a conventional H_2S donor
2. ZJ802 is capable of reducing blood pressure in anaesthetised mice
3. ZJ802 is an antioxidant
4. ZJ802 is anti-inflammatory as demonstrated by its ability to reduce lung and liver MPO activity and plasma IL-6 in an *in vivo* mouse model of endotoxic shock.

ZJ802 an organosulfur compound was synthesised as a putative H_2S releasing agent. In this Chapter, ZJ802 was shown to reduce blood pressure in the mouse *in vivo*. ZJ802 also displayed antioxidant properties and produced a concentration dependent reduction in nitrate/nitrite levels in RAW264.7 cells challenged with LPS. In addition, ZJ802 pretreatment was shown to significantly ($P<0.05$) reduce MPO activity in lung and liver of LPS injected mice. This anti-inflammatory effect was further evidenced by a reduction in plasma IL-6 in this *in vivo* model of endotoxic shock. However, ZJ802 injected into mice after the injection of LPS did not significantly reduce MPO activity in lung, liver or kidney.

6.3.1 Is ZJ802 a H_2S donor?

The release of H_2S from ZJ802 was detected by the DTNB assay and the ISE. However, H_2S was not detected from ZJ802 using the methylene blue colourimetric assay (Figure 6.2.1) and therefore this compound cannot be considered as a conventional H_2S donor.

The reason for the differences between the H_2S detection assays is unclear. It can be postulated that ZJ802 interrupts the formation of methylene blue and may be one reason why H_2S was not detected. The DTNB assay is a method used to quantitate thiol (-SH) groups on compounds (Sedlak and Lindsay 1968). As ZJ802 does not have an obvious thiol group on the parent molecule itself, it is likely that ZJ802 breaks down to a smaller thiol containing moiety which the DTNB assay subsequently detects. Although both the ISE and the DTNB assay could both detect "H_2S" (if it is indeed H_2S), the absolute concentration of H_2S detected between these two methods were strikingly different. ZJ802 (1 mM) was shown to release approximately ~330 µM H_2S measured by the DTNB assay, whereas the ISE suggested ~10 µM H_2S at the same concentration of ZJ802. There are several problems with using the ISE such as drift, harsh antioxidant buffer conditions (pH 13) and changes in voltage with background (see Chapter 3, discussion 3.3.4) and therefore whether the ISE is measuring a change in sulfide levels is questionable. On the contrary, if a concentration of 300 µM H_2S was achieved it would generate a detectable odour, however, this was not the case. Therefore whether the DTNB assay is measuring H_2S or an –SH group on an alternative breakdown product of ZJ802 is yet unclear.

On injecting ZJ802 into anaesthetised mice, ZJ802 (50 mg/kg, i.v.) induced an almost instantaneous fall in blood pressure that was longer lasting than the transient drop in blood pressure induced by NaHS (Figure 6.2.7). Whether ZJ802 decomposes into a vasodilator product, possibly H_2S or perhaps another biologically active sulfur-containing species, or directly induces vasodilation is unknown. Interestingly, organosulfur compounds such as allicin, the active ingredient in garlic, has been shown to reduce blood pressure in humans (Ried, et al. 2008). Garlic is a source of H_2S

(Benavides, et al. 2007) and/or sulfane sulfur (Iciek, et al. 2009), both of which may carry out some of its biological actions. Interestingly, although allicin has been implicated to be a source of H_2S (Benavides, et al. 2007), solubilising allicin does not result in the characteristic H_2S odour, but it has been suggested that in presence of endogenous thiols such as GSH, allicin decomposes to H_2S (Benavides, et al. 2007). Indeed, further experiments would be necessary to examine whether ZJ802 releases H_2S *in vivo*, by determining plasma H_2S over a number of time points, and/or whether ZJ802 releases H_2S *in vitro* on incubation with reducing agents e.g. GSH, or biological material such as liver or blood plasma.

It is possible the blood pressure effects of ZJ802 are due to a number of mediators. It is therefore necessary to carry out *ex vivo* blood vessel caliber studies with the aid of antagonists that inhibit the action of vasoactive mediators: H_2S (glibencamide, K_{ATP} channel blocker), prostaglandins (NSAIDS), $^{\bullet}NO$ (L-NAME NOS inhibitor or haemoglobin $^{\bullet}NO$ scavenger), cyclic GMP (ODQ, soluble guanylate cyclase inhibitor), cyclic AMP (SQ23356, adenylate cyclase inhibitor), endothelium-derived hyperpolarising factor (apamin/charybdotoxin, inhibitors of small/large K_{Ca} channels) or K^+ channels (tetraethylammonium, non-selective K^+ channel blocker).

Overall, ZJ802 does not appear to be a conventional H_2S donor like NaHS or GYY4137. However, it is possible ZJ802 may release H_2S. Indeed, further studies are required to confirm this.

6.3.2 ZJ802 in inflammation

Although the H_2S releasing potential of ZJ802 is unclear, this compound exhibited comparable, if not more potent, antioxidant properties to GYY4137 and NaHS (Figure 6.2.8). Antioxidants are important in limiting damage induced by free radicals and may therefore play an important role in limiting damage to healthy cells during the inflammatory process.

Antioxidants have been demonstrated to have a number of anti-inflammatory effects (Fang, et al. 2002). Vitamin E has previously been shown to inhibit NF-κB activation (Suzuki and Packer 1993, Faruqi, et al. 1994). Furthermore, plant flavonoids also exhibit anti-inflammatory properties (Kim, et al. 2004) and have been shown to inhibit COX-2 and iNOS expression from inflammatory cells stimulated with LPS (Liang, et al. 1999, Chi, et al. 2001, Raso, et al. 2001). The antioxidant properties of ZJ802 prompted further studies to determine whether this would be a pharmacologically viable compound in inflammation.

ZJ802 pre-treatment significantly reduced MPO activity in lung and liver tissue of LPS-treated mice. In addition, ZJ802 significantly reduced pro-inflammatory cytokine IL-6 but not TNF-α in the plasma. The reason why ZJ802 significantly reduced IL-6 but not TNF-α is unknown. Plasma TNF-α is transiently up-regulated for <120 min following a pro-inflammatory stimulus *in vivo* (Barsig, et al. 1995, Amiot, et al. 1997). It is possible that time point utilised in this study had either past or preceded the optimal peak TNF-α plasma levels and may be one reason why large differences occurred in measuring plasma TNF-α between mice.

Interestingly, ZJ802 post-treatment *in vivo* had no effect on tissue MPO activity compared to control. In contrast, GYY4137 has previously been demonstrated to

significantly reduce the LPS-induced hypotension in rats as well as plasma TNF-α, IL-1β, IL-6 and lung MPO activity when administered post-LPS insult (Li, et al. 2009b). Whether ZJ802 will be therapeutically viable in other models of inflammation requires further investigation.

6.3.3 Summary and conclusion

There are several potential problems with fast releasing H_2S drugs, such as NaHS, most notably the effect on cell viability (see Chapter 5). The need for more slow releasing H_2S donors, like GYY4137, may offer an alternative means of increasing our understanding of H_2S in biological systems. Through analysing a cohort of potential viable H_2S releasing compounds (not reported in this thesis), a potent antioxidant was discovered, ZJ802. This compound also exhibited anti-inflammatory properties *in vivo*. However, the anti-inflammatory effect of ZJ802 is not apparent when injected after LPS (i.e. post-treatment). Whether any apparent beneficial effects of this compound as an anti-inflammatory agent will be therapeutically viable is unknown as is the toxicology of this compound.

CHAPTER 7: Overview and Conclusion

7. Introduction and summary of thesis

The first half of this thesis attempts to elucidate the roles of endogenous H_2S in the vasculature that is, whether H_2S release from HUVEC is Ca^{2+} dependent and if signalling crosstalk exists between $^{\bullet}NO$ and H_2S. The second half of this thesis utilises pharmacological and biochemical techniques to examine the potential therapeutic benefits of GYY4137 in models of inflammation and to characterise a novel compound, ZJ802.

i) Is H_2S as an EDRF?

Evidence suggests that H_2S, like $^{\bullet}NO$, may be an EDRF (Yang, et al. 2008). Indeed it has previously been demonstrated that both HUVEC and BAEC contain CSE protein and that stimulation of these endothelial cells with a Ca^{2+} mobilising stimulus (A23187 and MCh) results in the release of detectable amounts of H_2S into the cell culture medium (Yang, et al. 2008). If H_2S is an EDRF, a number of exciting new questions could be answered, for example: Would H_2S released into the medium from stimulated HUVEC relax vascular smooth muscle cells *in vitro*? ; Is H_2S released from *ex vivo* blood vessels in the presence of a Ca^{2+} stimulus? ; Would an increase in plasma H_2S be observed upon a Ca^{2+} mobilising stimulus/drug *in vivo*? ; Would other physiological means, such as flow, induce the release of H_2S from cultured endothelial cells? Finally, does 'chronic' incubation with L-NAME/L-argnine free medium or PAG/L-cysteine free medium, result in compensatory mechanisms leading to the release of H_2S or NO respectively from endothelial cells? In order to answer these questions it is necessary

to measure detectable amounts of H_2S release from endothelial cells. However, in Chapter 3 and in contrast to a previous study (Yang, et al. 2008), H_2S was not detectable in the media of cultured endothelial cells or RAW264.7 macrophages, in response to a Ca^{2+} stimulus or from LPS respectviely (Chapter 3). Moreover, incubating liver homogenates with Ca^{2+}, Ca^{2+} chelating agents or Ca^{2+}/CaM antagonists did not alter H_2S synthesising activity, suggesting CSE/CBS are Ca^{2+} independent. One of the limitations of the studies presented within this thesis may have been that the H_2S measuring methods may have not been sensitive enough to measure H_2S elaboration from cultured cells. Evidence suggests that the release of endogenous H_2S is likely to be below or within the nM range (Olson 2009), whilst in the current study the limit of detection of ISE and methylene blue was 10 µM and 3 µM H_2S respectively. Whilst the same techniques were identical to that used in Yang et al., 2008, they did not provide details relating to the limit of H_2S detection, or the absolute concentrations of H_2S released within their study. Interestingly a time-dependent drift was observed ultilising the ISE in cell culture medium. Moreover, changes in background unrelated to H_2S release could also alter voltage differences utilising this method of H_2S detection. Thus, it may be possible that the apparently detectable H_2S in the study by Yang et al., 2008, may have been non-selective effect of the ISE.

It was also interesting to note that MCh did not cause a measurable rise in intracellular Ca^{2+} in endothelial cells in the current study. The previous study did not directly demonstrate MCh could increase in intracellular Ca^{2+} in their endothelial cell lines (Yang, et al. 2008), questioning whether the release of 'H_2S' from endothelial cells result from increasing intracellular Ca^{2+} concentrations. In contrast to the previous study (Yang, et al. 2008), neither CSE nor CBS protein was detected in HUVEC, possibly

due to method sensitivity or differences in antibody utilised. However, CSE and CBS mRNA was detected in HUVEC suggesting that H_2S may potentially act as an EDRF. If H_2S were to act as an EDRF, it is likely the methods utilised may not be sensitive or reliable enough to detect *de novo* H_2S release in cell culture.

ii) $^{\bullet}NO$ and H_2S crosstalk

Although unable to measure H_2S production from endothelial or RAW264.7 cells in culture, it was possible to elucidate crosstalk between endogenous $^{\bullet}NO$ and H_2S by measuring H_2S synthesising enzyme activity from organs of genetically modified mice lacking iNOS and eNOS. The examination of 'gas-enzyme' and 'gas-gas' crosstalk between $^{\bullet}NO$ and H_2S has previously been examined. For the first time, this thesis demonstrates a direct ('enzyme-enzyme') crosstalk between CSE and eNOS (Chapter 4). This thesis has demonstrated that knocking out eNOS, but not iNOS, results in an increase in H_2S synthesising activity in the liver, stomach and small intestine. This suggests that the actions of H_2S may be able to compensate for the loss of $^{\bullet}NO$. Indeed, further functional studies are required to confirm these new exciting findings.

During the process of examining H_2S synthesising activity from a number of tissues, it was noted that the H_2S synthesising assay was bias towards the activity of CSE. This was demonstrated by large detectable amounts of CBS protein with a lack of H_2S synthesising activity in mouse brain homogenates. This assay, therefore does not examine the total H_2S synthesising enzyme activity, as was initially predicted, and is a limitation to this study. Indeed, caution should be taken to interpret experimental data utilising H_2S synthesising activity, particularly from literature where several 'H_2S synthesising enzyme' assays have been utilised (Stipanuk and Beck 1982, Abe and

Kimura 1996, Li, et al. 2005, Singh, et al. 2009). Recently, lanthionine was suggested to be a biomarker of CBS (Singh, et al. 2009). Although CSE is also capable of synthesising lanthionine this reaction is negligible compared to CBS (Chiku, et al. 2009, Singh, et al. 2009). Therefore measuring lanthionine as an endpoint marker, rather than H_2S, may be an alternative means to measure the activity of CBS *in vitro*.

iii) GYY4137 vs NaHS in inflammation

The use of pharmacological tools such as H_2S donors is one approach to elucidating the biological roles of H_2S. Both H_2S donors and H_2S inhibitors have been demonstrated to exert both pro- and anti-inflammatory actions in various models of inflammation (Chapter 5). The controversy in the literature may be caused by number of factors including: the use of CSE/CBS inhibitors or H_2S donors, the concentration of H_2S, the time of administration, the stimulus utilised, the cell type, animal species, and the *in vitro* or *in vivo* conditions. Moreover, NaHS in the current study appears to exert a biphasic inflammatory effect which causes added complication to an already ambiguous situation. In addition, the effects of NaHS, particularly at millimolar concentrations, may be accounted for by its potential toxicity. To date, studies investigating the effects of the novel slow releasing H_2S donor GYY4137 have been promising. This compound has shown to exert anti-inflammatory/protective effects in a model of endotoxic shock *in vivo* (Li, et al. 2009b), LPS stimulated RAW264.7 cells *in vitro* (Whiteman, et al. 2010c), oxidative stress induced by H_2O_2, 4-HNE (4-hydroxynonenal) and SIN-1 (3-morpholinosydnonimine) on human chondrocytes *in vitro* (Fox, et al. 2011), and this thesis supports a role for an anti-inflammatory nature for GYY4137 (Chapter 5). The need for more slow releasing H_2S donors is necessary to

confirm a beneficial role for H_2S in inflammation. Indeed, whether slow releasing H_2S donors will be of therapeutic benefit is currently unknown due to limited data, but appears to be highly feasible (see later).

iv) Organosulfur compounds

In an attempt to assess a library of drugs that may potentially release H_2S, an antioxidant was discovered, ZJ802. This compound exhibited greater antioxidant potential than NaHS and GYY4137 and was shown to also exert some beneficial anti-inflammatory effects *in vivo*. Interestingly, other organosulfur compounds such as allicin and sulforaphane have antioxidant properties and may potentially exert their effects through the release of H_2S (Chapter 6). Whether ZJ802 releases H_2S is unclear as is the toxicological profile of this compound.

7.1. Methods used to measure H_2S

Plasma H_2S and H_2S synthesising enzyme activity is elevated in a number of inflammatory diseases (Bhatia, et al. 2005, Li, et al. 2005, Whiteman, et al. 2010b). As a result H_2S has been suggested to act as a biomarker for a number of conditions such as atherosclerotic peripheral arterial disease, (Kevil 2011) and asthma (Wang, et al. 2011a). However, the sensitivity and selectivity (for free, acid-liable or protein bound sulfur) of the different methods used to measure H_2S is a limitation. The inconsistent plasma levels of H_2S, ranging from 10-300 μM, likely results from different methods used to measure H_2S (Whitfield, et al. 2008). The predominant methods used to measure H_2S in the literature are the methylene blue colourimetric method and the ISE. The harsh chemical conditions exerted by both methods result in the release of H_2S from acid-liable sulfur or protein bound sulfur, which may artificially elevate the

concentration of H_2S detected (Chapter 3). In order to fully understand the physiological role of endogenously produced H_2S and the effects of H_2S donors, a reliable, sensitive method to directly measure H_2S is required. Data presented within this thesis indicates that there may be some inherent problems with the methods that are currently available and this in turn may explain some of the variable/conflicting data published within the literature. This limitation of H_2S direct measurement has prompted a number of alternative methods for measuring H_2S as discussed below.

The polarographic H_2S sensor is an amperometric microsensor that estimates H_2S gas indirectly from pH (Doeller, et al. 2005, Olson 2009). The polargraphic sensor has a reported sensitivity of 14 nM H_2S gas and 100 nM total sulfide (Whitfield, et al. 2008) contrasting the current study where the methylene blue and ISE method, in the current study, has a sensitivity of ~ 3 and 10 μM total sulfide respectively. Furthermore in contrast to the current methods which utilise harsh chemical conditions to 'trap' (or react with) H_2S, the polarographic sensor measures sulfide in real time (Whitfield, et al. 2008). Whether flaws exist with this microsensor remains to be elucidated.

Gas chromatography has a reported sensitivity in the nM range with 0.1 nM H_2S detected in blood and 15 nM H_2S in the gas space above brain homogenates incubated with 1 mM cysteine (Furne, et al. 2008). This method is sensitive and specific for measuring H_2S gas. However, in solution such as blood plasma, if H_2S were to be generated endogenously, other forms of H_2S (e.g. HS^- and possibly S_2^-) are likely to exist in addition to molecular H_2S.

A group in Japan recently created a novel method to measure free H_2S using silver (Ag) particles combined with gas chromatography (Ishigami, et al. 2009). In this assay, Ag powder was incubated with the supernatant of tissue homogenates to

convert sulfide into silver sulfide (AgS) (Ishigami, et al. 2009). The Ag powder was extracted and incubated with thiourea/H_2SO_4 to induce the release of H_2S, which was subsequently quantitated using gas chromatography (Ishigami, et al. 2009). This method has the advantage, over conventional methods (methlyene blue) in that H_2S from acid-liable from tissues will not be released. However, in this study the authors utilised a strong alkali buffer (borate buffer, pH 9) to homogenise their tissues which may induce the release of H_2S from protein bound sulfur (Olson 2009). The reason for the use of alkali borate buffer is unclear, but may be utilised to preserve H_2S in the S_2^- form. Whether another buffer within a physiological pH range would be suitable for this assay, is something that requires further consideration.

In a recent study, a sulfide derivatisation method was utilised to measure blood sulfide in human volunteers injected with Na_2S (Toombs, et al. 2010). In this protocol, blood sulfide was incubated with monobromobimane to form sulfide dibimane, a fluorescent product which was extracted and separated by high-performance liquid chromatography (HPLC) and the fluorescence quantitated (Toombs, et al. 2010). This method can detect 0.4-0.9 µM sulfide in baseline rat blood (Wintner, et al. 2010). The polarographic sensor and the monobromobimane assay appear to correlate when measuring H_2S in buffer and human plasma spiked with Na_2S *in vitro* (Wintner, et al. 2010). However, the monobromobimane assay detects significantly more H_2S than the polarographic sensor in whole blood spiked with Na_2S *in vitro* (Wintner, et al. 2010). Moreover, the measurement of H_2S from whole blood samples obtained from rats injected with Na_2S (60 mg/kg/h) using the two independent methods demonstrated marked differences in absolute H_2S concentrations (Wintner, et al. 2010). Less than 1 µM H_2S was detectable by the polarographic sensor and up to 30 µM H_2S was

measured using the monobromobimane assay (Wintner, et al. 2010). Interestingly, in another study within 5 min of injecting radiolabled sulfide ^{35}S (i.v.) into rats 70% of the ^{35}S was bound to proteins (half to plasma proteins and half to red blood cells) (Curtis, et al. 1972). The results from the latter study may suggest that upon administering H_2S *in vivo*, H_2S is quenched by proteins in the blood and any free H_2S left will be minimal. Indeed, this may be a reason why injecting NaHS into animals only results in a transient (~30 sec) (Zhao, et al. 2001) drop in blood pressure. These latter studies may suggest that the monobromobimane assay is thus measuring protein bound sulfur in addition to free H_2S.

Overall, it is likely that most of the methods used to measure H_2S are not just measuring free H_2S, but also sulfur from a source of protein. Therefore it is necessary to utilise more than one method when measuring H_2S, or indeed not measuring endogenous blood or tissue H_2S. There is currently no gold standard for measuring H_2S and acknowledgement of the limitations associated with each method is necessary before making a full conclusion regarding concentrations of H_2S. The most accurate measurement of H_2S so far is probably the polarographic sensor which can measure free H_2S in real time without chemical modification. Interestingly, this device cannot detect H_2S in the plasma (Whitfield, et al. 2008) and this questions whether H_2S exists as a freely circulating endogenous signalling molecule (discussed later).

7.2. Biomarkers for H_2S

The methods for measuring H_2S are not perfect. H_2S itself is unstable (liable to phase transition) if not chemically modified. Biomarkers may be an alternative method to measure levels of H_2S. H_2S is endogenously detoxified to sulfate and may be utilised as a biomarker for H_2S. However, sulfate in the urine may be derived from the

oxidation of cysteine by cysteine dioxygenase to form cysteine sulfinic acid (Wainer 1965). The transamination of cysteine sulfinic acid results in the production of β-sulfinylpyruvate which is cleaved to form pyruvate and sulfite (Singer and Kearney 1956). Sulfite is then catalysed to sulfate by sulfite oxidase and excreted in the urine (Singer and Kearney 1956).

Urinary thiosulfate levels have previously been used as a non-specific biomarker for the physiological production of H_2S (Belardinelli, et al. 2001) and for individuals over-exposed to H_2S (Kage, et al. 1997). Indeed, the primary route by which H_2S is removed is predominately through conversion to sulfate or thiosulfate through the action of mitochondrial enzymes quinone oxidoreductase, sulfur dioxygenase and a sulfur transferase (Hildebrandt and Grieshaber 2008). However, thiosulfate is also a by-product of cysteine and methionine metabolism (Gast, et al. 1952) where cysteine can be directly converted to 3-mercaptopyruvate by CAT, which is subsequently converted to thiosulfate by 3-MST (Wrobel 2001, Mikami, et al. 2011a, Tanizawa 2011). Although, sulfate and thiosulfate may not be entirely specific for H_2S, currently no studies have demonstrated whether plasma H_2S levels correlate with urinary/plasma sulfate or thiosulfate levels and/or whether dietary intake of cysteine correlates with plasma H_2S levels *in vivo*.

Lanthionine and homolanthionine has been suggested to act as a potential biomarker for H_2S production (Chiku, et al. 2009). However, it is possible external factors e.g. lanthionine antibiotics (Parisot, et al. 2008) or internal infections from gram negative bacteria (Wadman, et al. 1978) may influence urinary lanthionine levels. Interestingly, homolanthionine is elevated in the urine of patients with homocystinuria (Perry, et al. 1966), which implies *dysfunctional* CBS may lead to elevated

homolanthionine in the urine. These authors suggested that the formation of homolanthionine was due to the condensation of homocysteine and homoserine (Perry, et al. 1966). Therefore urinary (and possibly plasma) homolanthionine may not be a suitable biomarker for CSE/CBS/H_2S activity *in vivo*.

7.1. H_2S as an endogenous signalling mediator

There has been some debate as to whether H_2S is an endogenous signalling mediator. Indeed, the methods used to detect H_2S have limitations and whether free H_2S exists in the blood is controversial. Indeed, at some of the concentrations reported in blood plasma (~40 μM) one would expect the distinct smell of H_2S to be clearly detectable. However, this is not the case. Moreover, the rapid binding of sulfide to blood proteins (Curtis, et al. 1972) as well as the short lasting action of NaHS on blood pressure (Zhao, et al. 2001) may suggest that free sulfide does not circulate in blood. However, H_2S may exert its actions through other mechanisms. H_2S can modify the functions of proteins through s-sulfhydration, that is, the reaction with a disulfide bond to form a persulfide R-SSH and thiol -SH (Mustafa, et al. 2009, Kabil and Banerjee 2010). It has also been suggested that the actions of sulfide are conducted by sulfane sulfur (Toohey 2011). Sulfane sulfur atoms are reactive sulfur atoms with a valency of 0 or -1 and do not exist in the free form but are instead found covalently bound to other sulfur atoms (Westley, et al. 1983, Toohey 1989, Iciek and Wlodek 2001). Such sufane sulfur compounds include thiosulfate, elemental sulfur S^o, persulfides R-SSH, disulfides R-SS-R and polysulfides R-S_n-R (Toohey 2011). The addition of exogenous H_2S to a system also introduces sulfane sulfur which can itself induce the formation of persulfides from protein disulfide bonds (Toohey 2011). Moreover, CSE, 3MST and rhodanese

contribute to the formation of sulfane sulfur compounds during anaerobic cysteine metabolism (Iciek and Wlodek 2001). Sodium thiosulfate has been described as an antioxidant and demonstrated to delay the vessel calcification process in humans (Adirekkiat, et al. 2010). H_2S can indirectly increase endogenous thiosulfate levels (Hildebrandt and Grieshaber 2008). However, thiosulfate itself is also capable of increasing endogenous H_2S (Sen, et al. 2008, Mikami, et al. 2011a). Whether H_2S or thiosulfate is the active molecule requires further investigation. Indeed, further studies are required to determine the molecular mechanism by which H_2S acts and whether the actions of CSE and H_2S are through the production and action of s-sulfhydration, sulfane sulfur or possibly thiosulfate.

Although it is questionable whether H_2S is a free circulating gaseous mediator, the small diffusible membrane permeable property of H_2S makes it a suitable endogenous signalling molecule. H_2S can be released from bound sulfur sources in the presence of physiological sources of GSH (Ishigami, et al. 2009), and may therefore act as a signalling molecule during oxidative stress. Furthermore, the transulfuration pathway is intimately connected to the GSH synthesising pathway, where L-cysteine is the rate limiting substrate for the synthesis of GSH (Shackebaei, et al. 2005). This means the transulfuration pathway, the predominant pathway for endogenous H_2S generation, may also be responsible for driving the production of GSH which itself may promote release of H_2S from protein bound sulfur sources. Interestingly, the transulfuration pathway is also up-regulated in response to oxidative stress (Persa, et al. 2004). Moreover, CBS is redox active, and oxidation increases its enzyme activity (Taoka, et al. 1998). Indeed, during oxidative stress, H_2S is capable of reacting with reactive oxygen species such as peroxynitrite *in vitro* (Whiteman, et al. 2004), and may

regulate endogenous levels of $^{\bullet}$NO through the formation of a biologically less reactive nitrosothiol (Whiteman, et al. 2006). Indeed, this thesis supports a role for H_2S as an antioxidant *in vitro* (Chapter 6) and it is possible H_2S acts as an endogenous signalling mediator during oxidative stress.

7.2. H_2S donors and inhibitors

H_2S donors and CSE/CBS inhibitors have been useful tools in understanding the biological actions of what is currently known about H_2S. CSE/CBS inhibitors elicit non-selective effects (Pfeffer and Ressler 1967, Tanase and Morino 1976, Burnett, et al. 1980, Loscher 1981, Hamel, et al. 1982), which has also been demonstrated in this thesis. The H_2S synthesising enzyme activity utilised in the current studies is bias towards CSE activity (Chapter 4), yet AOAA inhibitor of CBS, could almost abolish rat liver H_2S enzyme activity (Chapter 3) suggesting AOAA is non-selective.

There are several commercially available H_2S salts (e.g. NaHS, Na_2S, calcium sulfide CaS) and Lawesson's reagent. However, the purity of these H_2S donors is questionable. For example, the purity of NaHS is not usually greater than 70% and could also be another reason for the discrepancy in the literature when using H_2S donors *in vitro* and *in vivo* (Whiteman and Winyard 2011). As described earlier in Chapter 5 of this thesis, there are several problems with these donors, namely they can cause cell death (Yang, et al. 2004, Cao, et al. 2006, Yang, et al. 2007). Moreover, these salts probably do not mimic the slower biological release of endogenous H_2S. In contrast, the use of slow releasing H_2S donors may provide a better insight into the roles of endogenous H_2S and are less likely to induce cell death as a result of a rapid release of large concentrations of H_2S. Organosulfur compound, GYY4137 has been

shown, to date, to consistently exert an anti-inflammatory effect both *in vivo* and *in vitro* (Chapter 5). Therefore, the progressive release of H_2S from slow releasing donors, like GYY4137, may be of more therapeutic relevance and better tools to examine the effects of H_2S than are conventional H_2S salts.

The beneficial effects of slow releasing H_2S donors have been exemplified by H_2S-NSAID hybrids. ADT-OH (anethol trithione, 5-(4-hydroxyphenyl)-3H-1,2-dithiol-3-thione) is a H_2S donor and in combination with NSAIDs such as diclofenac results in the slow release of H_2S (Li, et al. 2007). Moreover, there is increasing evidence for a beneficial role for H_2S-NSAID hybrids in limiting gastrointestinal damage induced by NSAIDs (Distrutti, et al. 2006b, Fiorucci, et al. 2007, Li, et al. 2007, Wallace, et al. 2007a). So far, several ADT-OH-hybrid drugs have been produced, such drugs include: H_2S-NSAID hybrids (e.g. aspirin ACS14, indomethacin ATB343, diclofenac ATB337 or ACS15, mesalamine ATB429); H_2S anti-glaucoma hybrid (latanoprost-H_2S hybrid ACS67); and H_2S-phosphodiesterase hybrids (sildenafil ACS6). These H_2S hybrids have provided further insight of the benefits of H_2S administration and may provide an alternative means by which H_2S can enhance the efficacy/drug tolerability of currently available drugs for human consumption. However, it has been suggested that the beneficial effects of ADT-OH may be as a result of the dithiolethione moiety itself rather than the release of H_2S (Isenberg, et al. 2007), and is therefore an area that requires further evaluation.

Exogenous L-cysteine is thought to increase endogenous production of H_2S and may be an alternative means for producing H_2S. Cysteine analogues have been generated (e.g. S-allyl-l-cysteine SAC and S-propargyl-l-cysteine SPRC) (Wang, et al. 2009a). However, the beneficial effects of these cysteine analogues over L-cysteine

itself, is unclear. None-the-less, these analogues have been demonstrated to promote endogenous H_2S production (Wang, et al. 2009a) and have anti-cancer effects both *in vitro* and *in vivo* (Ma, et al. 2011). The addition of L-cysteine has previously been shown to up-regulate the expression of cysteine dioxygenase, the enzyme involved in taurine synthesis, and down-regulate γ-glutamylcysteine synthetase, the enzyme involved in GSH synthesis (Kwon and Stipanuk 2001). However, L-cysteine is the substrate for GSH production and may therefore potentially increase GSH production on increasing L-cysteine availability. Whether the effects of adding L-cysteine are due to changes in the anti-oxidants GSH or taurine, and/or H_2S is more difficult to decipher. Therefore although added cysteine may lead to increases in endogenous H_2S generation, other antioxidant systems are also likely to change as a result.

Recently, a novel H_2S donor has been described, based on the structure of N-(benzoylthio)benzamide (Zhao, et al. 2011). These H_2S donors release H_2S in the presence of excess cysteine, which provides a mechanism for the controlled release of H_2S (Zhao, et al. 2011). However, whether enough free cysteine exists *in vivo* to induce the release of H_2S from these of N-(benzoylthio)benzamide based H_2S donors requires further study (Zhao, et al. 2011).

Meta analysis of clinical trials has demonstrated the benefits of using garlic derived compounds on blood pressure regulation in humans (Ried, et al. 2008). The mechanism behind the beneficial effects of garlic is unclear. However, garlic organosulfur compounds (diallyl disulfide, diallyl trisulfide) have been shown to release H_2S in the presence of red blood cells under anoxic conditions (Benavides, et al. 2007). Whether these reactions take place *in vivo* require further examination. Indeed, other organosulfur compounds such as sulforaphane have cardiovascular and anti-

inflammatory benefits (Vazquez-Prieto and Miatello 2010). Indeed, the potential benefits of oraganosulfur compounds have been demonstrated in Chapter 6 of this thesis. Whether these compounds are converted to H_2S endogenously require further investigation.

Overall there are several available compounds that can be utilised to examine the effects of H_2S *in vitro* and *in vivo*. It is likely there will be increasing interest in the generation of slow releasing donors in the future. The use of different organosulfur compounds as a basis for H_2S generation *in vivo* may be an ideal starting point for the generation of future slow releasing H_2S donors.

7.3. Future for H_2S and the cardiovascular field

Most of the actions of H_2S in the cardiovascular system have focused on a very pharmacological approach to assessing the roles of H_2S in the vascular system, using H_2S donors or inhibitors of CSE/CBS. However, the role for endogenous H_2S signalling within the cardiovascular system remains largely unexplored. Although CSE/CBS inhibitors have provided insight into the endogenous roles of H_2S, the effects of these inhibitors remain speculative due to their non-selective effects. However, transgenic mice will probably be a useful tool to increase knowledge regarding the function of endogenous H_2S. However, KO mice have their flaws, such as potential compensatory mechanisms and in particular regard to CSE/CBS KO mice, the increase in plasma homocysteine. None-the-less, $CSE^{-/-}$ KO mice have a reduced plasma H_2S and have provided an alternative insight to the endogenous functions of H_2S dispite the setbacks. Indeed the development of $CSE^{-/-}$ mice has demonstrated a role for endogenous H_2S in blood pressure regulation (Yang, et al. 2008), angiogenesis

(Papapetropoulos, et al. 2009), O_2 sensing in the carotid body (Peng, et al. 2010), vascular remodelling (Yang, et al. 2010), and in the development of diabetes (Yang, et al. 2011b). Moreover, mice with a targeted over expression of CSE in the cardiac tissue are protected against left ventricular structural and functional impairment which may suggest a role for endogenous H_2S in cardiac remodelling (Calvert, et al. 2010). To provide further evidence for a role of CSE in cardiac remodelling, it would be interesting to examine whether $CSE^{-/-}$ mice would be more prone to heart failure.

In terms of inflammation, it would be interesting to examine whether differences in disease severity exist between $CSE^{-/-}$ and WT mice. The role of H_2S in nociception is unclear and these KO mice may also be a useful tool to increasing the understanding of endogenous H_2S in models of pain. There has been much interest in H_2S-releasing NSAIDS and ability to promote gastric healing compared to the conventional NSAIDS. Indeed, it could be predicted that the extent of gastric damage/bleeding would be reduced in mice with a targeted over expression of CSE or CBS. Slow releasing H_2S donor GYY4137 has been demonstrated to have anti-cancer effects as demonstrated by its ability to selectively induce apoptosis in cancer cell lines (e.g. HeLa, cervical carcinoma) but not non-cancer cell lines (e.g. WI-38, human fibroblast) (Lee, et al. 2011), and this questions whether low concentrations and slow rates of endogenous H_2S production may also elicit anti-cancer effects. Indeed, it could be hypothesised that $CSE^{-/-}$ mice may be more prone tumour progression in cancer models.

$ApoE^{-/-}$ injected with NaHS over 10 weeks exhibit reduced atherosclerotic plaque formation (Wang, et al. 2009b). It would be interesting to assess whether $ApoE^{-/-}$ mice also have a reduced H_2S enzyme activity. Moreover, it could be predicted that

crossbreeding $CSE^{-/-}$ with $ApoE^{-/-}$ mice would accelerate vascular calcification and the atherosclerotic process and increase the risk of myocardial infarction and/or stroke. Indeed it would also be interesting to explore whether a targeted over expression of CSE in vascular endothelial cells would attenuate the atherosclerotic process in $ApoE^{-/-}$ mice.

The general impression is that CBS is the predominant source of H_2S in the central nervous system and CSE in the peripheral systems. Both CBS and CSE mRNA has been demonstrated to be expressed in HUVEC (Chapter 3). It is likely that both CBS and CSE work together to generate H_2S. Indeed CBS is needed for the generation of cystathionine, a substrate of CSE, which in turn catalyses its conversion to cysteine. Although, it should be noted that both CSE and CBS appears to be promiscuous for their substrates (Singh, et al. 2009), therefore it is possible these enzymes can synthesise H_2S independently of each other. CBS is regulated by SAM and in the presence of this allosteric regulator may be the predominant source of H_2S (Singh, et al. 2009). However, the distribution and concentration of SAM throughout bodily tissues have not been determined and is an area that requires further consideration in order to examine whether CSE or CBS is the predominant source of H_2S in various organ systems.

Apart from the transulfuration pathway of H_2S biosynthesis, the CAT/3MST pathway may also be a source of H_2S. Whether the CAT/3MST enzymatic pathway occurs at physiological pH requires further investigation. None-the-less, the global enzyme expression and activity of the CAT/3MST pathway of H_2S generation has not been determined and is an area that requires further work. Moreover, the subcellular

generation of H_2S, either the cytoplasm or mitochondria, through this pathway has not been determined.

This thesis has explored the potential interaction between $^{\bullet}NO$ and H_2S using $eNOS^{-/-}$ mice. Whether chronic administration of L-NAME in rodents would increase H_2S enzyme activity is an alternative method to assess potential crosstalk between these two gaseous mediators. Other than $^{\bullet}NO$ and H_2S, CO is another gasotransmitter and although the actions of CO was not examined in this thesis, it is an area that requires further exploration. Indeed, it would be interesting to explore whether $CSE^{-/-}$ KO mice have increased total NOS or HO activity, or whether HO KO mice also have increased NOS or H_2S enzyme activity.

There are a vast number of possibilities that requires exploration regarding endogenous H_2S, particularly with the use of genetically modified mice. Indeed, this appears to be the most imminent way in assessing the role of endogenous H_2S particularly with the recent generation of $CSE^{-/-}$ mice.

7.4. Clinical perspective for H_2S

There has been increasing interest in the literature regarding the third gasotransmitter H_2S. H_2S has been demonstrated to have protective (and detrimental) effects in various pre-clinical models of inflammation, ischemia/reperfusion and remodelling. Already, within 10 years of active H_2S research, Na_2S has been administered into healthy human volunteers (total of 52 subjects, with 13 receiving placebo), as a phase I toxicology and tolerability trial (Toombs, et al. 2010). In this study, the administration of Na_2S (0.005 – 0.2 mg/kg i.v.) into humans did not exert any toxicological effects with no effects on the ECG (electrocardiogram), heart rate, blood pressure, temperature, respiration or

haematology, at the doses used (Toombs, et al. 2010). Although this gas did not have any toxicological effects in humans at the doses utilised (Toombs, et al. 2010) very little is known about the pharmacokinetics and pharmacodynamic profile of H_2S donors. As described earlier, the methods used to measure H_2S, although advancing, are still in its infancy. Moreover, there is increasing interest for the development of slow releasing H_2S donors, and much of their toxicology and pharmacokinetic/dynamic profile have not been thoroughly investigated *in vivo*. Overall, the phase I safety trial in humans (Toombs, et al. 2010) provides further reason to advance scientific knowledge and technology within the H_2S field.

Although the use of H_2S releasing drugs in the clinic are far from advance, H_2S itself is a potential biomarker for disease severity in conditions such as: coronary heart disease (Jiang, et al. 2005), rheumatoid arthritis (Whiteman, et al. 2010b), obesity and type 2 diabetes (Whiteman, et al. 2010a), peripheral arterial disease (Kevil 2011), and asthma (Wang, et al. 2011a). However, whether the methods used to measure H_2S truly reflect levels of H_2S or alternative forms of sulfur are currently uncertain.

7.5. Summary and conclusion

In summary, in this rapidly advancing field it is necessary not to overlook critical factors in methods used to measure H_2S, H_2S enzyme activity, and keep in mind the limitations of the agents used to inhibit enzyme activity or donate H_2S prior to making a full conclusion. There is the need for better methods of H_2S detection and selective pharmacological modulators of H_2S synthesising enzymes in order to advance the knowledge regarding the biological actions of endogenous H_2S. The development of genetically modified CSE mice will particularly be beneficial in advancing our

knowledge about endogenous H_2S produced by this enzyme. There appears to be a promising therapeutic role for exogenous H_2S, particularly in ischemia/reperfusion and gastric healing. Slow releasing H_2S donors such as GYY4137 will provide a greater insight into the benefits of H_2S donors in inflammation. As the therapeutic role for H_2S advances, there is the need for more detailed pharmacodynamic, pharmacokinetic and safety *in vivo* pharmacology data.

Overall, this thesis has: emphasised the need for better methods in the H_2S field particularly when assessing endogenous H_2S release; demonstrated a role for potential endogenous crosstalk between eNOS and CSE; provided further evidence for an anti-inflammatory role for slow-releasing H_2S donor GYY4137; and characterised a novel organosulfur compound with antioxidant and anti-inflammatory properties.

Appendices

Appendix A

Gel composition for SDS-PAGE. Volumes were made up to 10 ml using H_2O.

10ml/gel	**4% Stacking Gel**	**7.5% Resolving Gel**	**12% Resolving Gel**	**15% Resolving Gel**
Buffer	2.5ml 1.0M Tris pH 6.8 (Final: 0.125mmol/L Tris-HCl, 0.1% SDS, pH 6.8)	2.5 ml 1.5M Tris pH 8.8 (Final: 0.375mmol/L Tris-HCl, 0.1% SDS, pH 8.8)	2.5 ml 1.5M Tris pH 8.8	2.5 ml 1.5M Tris pH 8.8
Sodium dodecyl sulphate (SDS, 10% w/v)	100 µl	100 µl	100 µl	100 µl
Acrylamide	1.30 ml	2.5 ml	4 ml	5 ml
Ammonium Persulphate (APS, 10% w/v)	100 µl	50 µl	50 µl	50 µl
N N N’ N’-Tetramethylethylene-diamine (TEMED)	10 µl	5 µl	5 µl	5 µl

Laemmli sample buffer (2x)

	Volume
Tris-HCl 0.5M	2.4 ml
SDS	800 mg
Glycerol	4 ml
Bromophenol blue (0.01% w/v)	100 µl
β2-mercaptoethanol	1 ml
H_2O	2.5 ml

Appendix B

Western blot lysis buffer (Final concentration: EDTA 5mM, NaCl 150mM, Tris HCl 50 mM, Triton x-100 1%, SDS 1%, PMSF 1mM)

	Volume (µl)
EDTA (0.5 M)	50
NaCl (5 M)	150
Tris HCl (1 M)	250
Triton x-100 (10% v/v)	500
SDS (10% w/v)	500
Phenylmethanesulfonyl fluoride (PMSF, 100 mM)	50
Protease inhibitor cocktail (Sigma)	10
H_2O	3490

Appendix C

Primary antibodies (5% w/v non-fat milk, PBST)

Primary antibody	**Dilution**	**Origin/type**	**Source**
β-actin (42 kDa)	1:5000	Mouse polyclonal	Sigma (A5441)
α-tubulin	1:2000	Rabbit polyclonal	Cell Signaling Technology (#2144)
CSE (CTH) (43 kDa)	1:2000	Mouse monoclonal	Abnova (H00001491-M01)
CBS (75 kDa)	1:2000	Rabbit polyclonal	Abnova (H00000875-D01P)
COX-2	1:2000	Rabbit polyclonal	Cayman (#160106)
IκB-α	1:2000	Rabbit polyclonal	Cell signalling (#9242)
VCAM-1	1:500 (5% w/v albumin, PBST)	Goat polyclonal	R&D systems (BBA19)
ICAM-1	1:500 (5% w/v albumin, PBST)	Goat polyclonal	R&D systems (BBA17)

Secondary-HRP linked antibodies (5% w/v non-fat milk, PBST)

Secondary antibody	**Dilution**	**Source**
Rabbit anti-mouse polyclonal	1:5000	Sigma
Goat anti-rabbit polyclonal	1:2000	Cell Signaling Technology (#7074)
Rabbit anti-goat	1:5000 (5% w/v albumin, PBST)	Sigma (A5420)

Appendix D

Primers for PCR

Human CSE (157 bp)	reverse 3’ GTGGCTGCTAAACCTGAAGC forward 5’ CACTGTCCACCACGTTCAAG
Human CBS (150 bp)	reverse 3’ TTGGGGATTTCGTTCTTCAG forward 5’ TCATCGTGATGCCAGAGAAG
Human α-tubulin (200 bp)	reverse 3’ CACGTTTGGCATACATCAGG forward 5’ GACCAAGCGTACCATCCAGT

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